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316. TEMPERATURE CONTROL IN THE H.T.S.T. PASTEURIZATION PROCESS

BY J. A. HALL, *Physics Division, National Physical Laboratory, Teddington*

(With 1 Plate and 16 Text-figures)

INTRODUCTION

Until 1941 the holder process of pasteurization of milk was the only one officially recognized. To conform to the official designation *pasteurized milk* it was necessary to heat for a period of 30 min. at a temperature not below 145° F. nor above 150° F., and to cool to a temperature not exceeding 55° F. (1). The object of these regulations was to encourage the treatment of raw milk under conditions such that all the common organisms pathogenic for man would be destroyed, and that by the reduction in numbers of non-pathogenic types increased keeping quality would be secured. Within these limits of temperature and time the extent of damage, physical and chemical, to the milk, is minimal (2).

For many years the process of High Temperature-Short Time (H.T.S.T.) pasteurization has been used in industry, and in 1941 the Milk (Special Designations) Regulations (3, 4) gave, provisionally, official sanction to this method. The conditions, however, differ materially from those for holder pasteurization in that the time of holding is reduced to a minimum of 15 sec., and the temperature increased to not less than 162° F. It is, moreover, stipulated that a flow-diversion valve to operate when the temperature in the holding section falls below 162° F. must be incorporated in the system. It is clear that several problems in connexion with temperature control and recording become more important in the H.T.S.T. process than they are in the holder process. Thus in the latter, under static conditions, temperature changes are most unlikely to be rapid and, given thermometric apparatus of a suitable type, a fall in temperature would be recorded. It may therefore be said that although great accuracy is desirable, reasonable fluctuations (± 1° F.) in temperature are unlikely to be disastrous. When the holding time is reduced to 15 sec. and the temperature raised to 162° F., rapid response to fluctuations in temperature in the milk in a flowing stream is essential. Moreover, much smaller changes in temperature must be recorded.

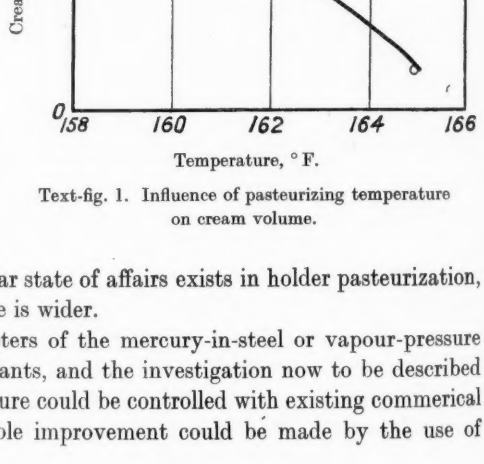
Since H.T.S.T. plants are now being equipped with temperature-control apparatus identical with that which is used for the holder process, it is clearly necessary to investigate its ability to meet the more stringent requirements, both as regards accuracy and quickness of response. Moreover, since the instruments have to withstand a continuously damp atmosphere, frequent splashing with liquid and considerable rough handling, exceptional robustness is also necessary.

The H.T.S.T. process may be considered in three stages, through which the milk passes continuously as it flows through the apparatus. The first stage is the heating of the milk to the pasteurization temperature. This heating is normally effected in a heat-exchanger

device. Here, accurate temperature control of the heating water is clearly necessary, but quickness of response in the thermometer is of secondary importance. The second, or 'holding' stage, takes place in a section of the apparatus which should be traversed by each particle of milk in a time of about 15 sec. without any serious drop in temperature. After this, the milk passes through another heat exchanger for the third stage—that of rapid cooling. The flow-diversion valve is used to reject any milk which may not have been held at a sufficiently high temperature throughout the holding period. This valve must be so arranged as to divert the flow of milk whenever a thermometer suitably placed in the holding section indicates a temperature below a predetermined safe value.

If we had an ideal thermometer, perfectly accurate and with no lag, and a valve capable of operating instantaneously, it would evidently be sound practice to place the thermometer at the end of the holding section and to set it to trip the valve at whatever temperature is selected as the low limit for effective pasteurization. But since no instruments are perfectly accurate and free from lag, it becomes necessary to place the thermometer bulb a certain distance upstream from this ideal point, so that the valve may be operated before any insufficiently heated milk reaches it. In such conditions, some allowance should be made for possible errors in the thermometer and for the slight drop in temperature which would normally be experienced in the flow between the thermometer and the valve, and the valve set to operate at a correspondingly higher temperature.

The regulations demand that the temperature shall not fall below 162° F. during the holding period, but other reasons make it desirable to pasteurize the milk at as low a temperature as possible. For example, impairment of the capacity of the cream to rise is notably increased with rise of temperature. Using a particular H.T.S.T. plant the approximate values of the cream volume obtained (expressed as a percentage of the volume of cream on the raw milk suitably treated before being set) are plotted in Text-fig. 1. The rapid drop in cream volume shows clearly that for optimum results the operating temperature should be controlled within the narrowest possible limits. A similar state of affairs exists in holder pasteurization, but the corresponding temperature range is wider.



Text-fig. 1. Influence of pasteurizing temperature on cream volume.

It is customary to rely on thermometers of the mercury-in-steel or vapour-pressure type for the control of pasteurization plants, and the investigation now to be described was to find out how closely the temperature could be controlled with existing commerical instruments and whether any appreciable improvement could be made by the use of thermocouples.

A. Existing commercial instruments

The properties of typical instruments of the mercury-in-steel and vapour-pressure types were examined under three heads: (i) accuracy and reproducibility of calibration,

(ii) thermometric lag, (iii) behaviour of control contacts (including the effect, if any, of the operation of the contacts on the accuracy of calibration of the instrument).

(i) *Accuracy*

Calibration corrections obtained by comparison with the standard thermometers of the National Physical Laboratory are given in Table 1.

Table 1. *Calibration corrections*

Instrument...	Mercury-in-steel recorder A		Mercury-in-steel recorder B		Vapour-pressure recorder		Vapour-pressure indicator	
Instrument reading °F.	Correction (°F.) Rising reading	Falling reading	Correction (°F.) Rising reading	Falling reading	Correction (°F.) Rising reading	Falling reading	Correction (°F.) Rising reading	Falling reading
130	+0·8	—	0·0	—	− 3·0	—	+0·8	+0·4
140	+1·0	—	−0·3	−1·1	− 2·5	—	+0·4	0·0
150	+0·8	—	−0·3	−1·1	− 2·0	—	0·0	−0·4
160	+0·8	−0·3	+0·3	−0·6	− 1·5	− 2·2	−0·5	−1·0
170	+0·9	−0·3	+0·7	−0·1	− 1·3	− 2·0	+1	−1
180	+0·3	−0·7	+0·6	−0·2	− 8	− 9	+4	+3
190	−0·5	−1·0	—	—	−15	−16	—	—
200	—	—	—	—	−10	−11	—	—

There are various points to be noted in this table of corrections. None of the instruments shows a better agreement between readings taken on rising and falling temperatures than 0·4° F., while in the case of the recorders the lowest difference is 0·7° F., the poorer performance being doubtless attributable to pen friction. The mercury-in-steel recorder A showed a difference slightly in excess of 1° F., and, in view of this fact and the results which were obtained in the tests on the behaviour of the control contacts, the makers supplied a second instrument, B, in which the pen-arm had been loaded so that gravity might help to overcome pen friction on a falling temperature. With this instrument the difference was 0·8° F. It should be noted also that the figures given in the table are mean values taken from smooth calibration curves and that *variations of the order of* 0·5° F. *were obtained between calibrations carried out under similar conditions on different days.* No investigation was made into the further errors which might be introduced by variations in temperature of the recorder movement and of the capillary.

It will be seen that the vapour-pressure instruments examined are liable to gross errors above 170° F. This type of thermometer normally has a scale which becomes more and more open as the temperature rises. In order to extend the scale, the two instruments used in this investigation were fitted with a spring device to restrict the movement of the pointer at temperatures above 170° F. In this way, the degree graduations just above 170° F. are spaced about four times as closely as those just below 170° F. Hence, if the spring does not begin to operate at exactly the right temperature (even though it may be correctly adjusted for strength), large errors are introduced. For example, if the spring comes into operation at 169° F. instead of 170° F., then the temperature will have risen to about 173° F. by the time the pointer reaches the 170° F. graduation. The spring of the recorder did not come into operation until about 175° F., while that of the indicator came into operation at slightly too low a temperature. Evidently such instruments cannot be regarded as even tolerably accurate at temperatures at which the spring is in action, which may be from a degree or so below the change in openness of the scale.

It is clear, therefore, that, even under ideal conditions, the accuracy of indicators or recorders of these two types cannot be regarded as better than $\pm 0{\cdot}5°$ F., while in commercial practice $\pm 1°$ F. would be a much more likely figure. For this accuracy, instruments should be individually calibrated and correction tables used. The accuracy may be still further impaired if the thermometer is required to do work by operating contacts for control or alarm purposes. This question will be discussed in section (iii) on the operation of contacts.

(ii) *Thermometric lag**

If a thermometer is placed in surroundings of which the temperature is different from that of the thermometer itself, the thermometer reading will change according to Newton's law of cooling:

$$\frac{dR}{dt} = \frac{1}{\lambda}(R-T), \tag{i}$$

where t = time in seconds, R = thermometer reading, T = temperature of surroundings, and λ = lag constant of thermometer in seconds.

When T is constant, the lag constant may be defined as the time in seconds taken to reduce the difference $(R-T)$ to $1/e$ (approximately 0·37) of its original value. When T is varying at a uniform rate, the reading (R) of the thermometer at any instant will be equal to the value which T had had λ sec. earlier. That is, the thermometer reading lags λ sec. behind the temperature of the enclosure.

The lag constant is not, in general, simply a function of the thermometer, but depends also on the character of the medium in which the thermometer is immersed and on its velocity past the bulb. However, if the velocity is infinite, the lag will be independent of the properties of the medium. With such a fluid as water or milk, any velocity in excess of 100 cm./sec. may be regarded as infinite. The velocity used in this investigation was not measured, but it was estimated to be at least 30 cm./sec., and this speed will, according to Harper, yield a value of λ only about 20% greater than that for infinite velocity. In H.T.S.T. pasteurization, a reasonable approximation to infinite velocity conditions may be assumed.

The method normally employed for the determination of λ is to plunge the thermometer into a constant-temperature bath giving the required conditions. Equation (i) then gives

$$(R-T) = (R_0-T)\, e^{-1/\lambda t}, \tag{ii}$$

where R_0 is the thermometer reading when $t=0$. Hence, by plotting $\log_e (R-T)$ against t, we get a straight line of which the slope is λ. The graphs which are given in this paper are plotted on a log scale to base 10 and, in consequence, the factor 0·434 has to be used to obtain λ from the slope.

The experiments were conducted in one of the water-baths used at the National Physical Laboratory for thermometer standardization. This bath is shown in section in Text-fig. 2. The observations were made by taking a cinematograph film of the movement of the recorder pen at a speed of about 8 or 16 frames per second, a stop-watch being included in the field to give a time scale. Pl. 1, fig. 1 is an enlargement from a single frame of one of the films. The data for plotting the lag curves were obtained from

* For a detailed discussion of lag phenomena, see D. R. Harper, *Thermometric Lag* (5).

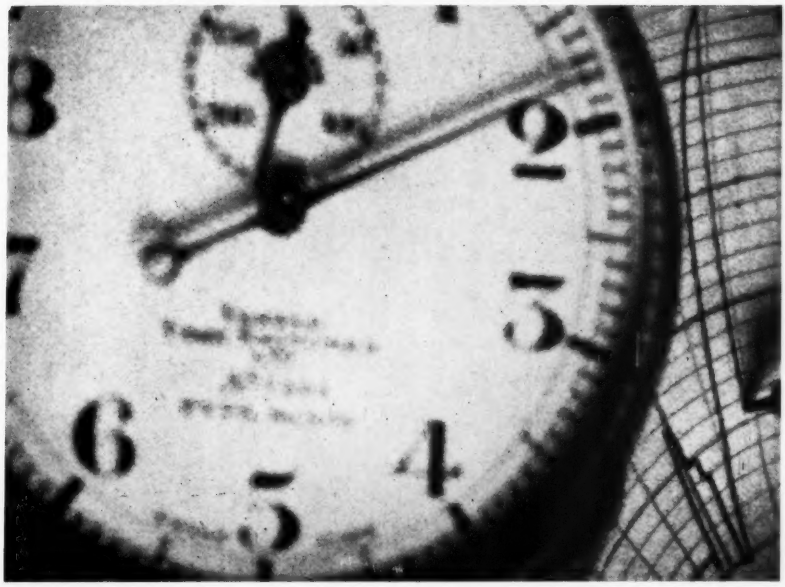


Fig. 1. Enlargement from cinematograph film of the lag constant determination.

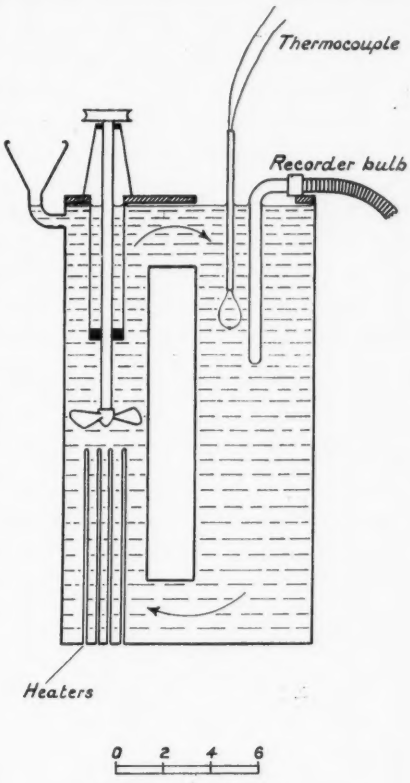
Fig. 2. Enlargement from cinematograph film of response to small temperature changes.

a frame by frame analysis of the films, the examination being made by using a projected image about 40 × 30 cm. in size.

The lag curves given in Text-fig. 3 were obtained with the mercury-in-steel recorder A and the vapour-pressure recorder, both of which had stainless steel bulbs. The curves were taken with the initial temperature of the bulb lower than that of the bath, so that they relate to rising readings of the thermometer. It will be seen that, with one exception, the mercury-in-steel curves approximate closely to the straight lines shown —·—·—, the slopes of these lines all giving values of λ between 3·0 and 3·2 sec. The curves given by the vapour-pressure recorder, on the other hand, are far from straight. The chain-dotted lines, taken in order, represent lags of 3·2, 3·6, 2·9 and 3·9 sec., but in each case the actual lag curve is initially steeper and finally less steep than this. In other words, the vapour-pressure thermometer will be 'quicker off the mark' but slower in reaching final equilibrium with the bath.

A possible explanation of this result is that the bulb of a vapour-pressure thermometer is partly filled with liquid and partly with vapour. The lag curve is thus the resultant of two different log relationships, one relating to liquid in steel and one to vapour in steel. This effect has been analysed by Harper in relation to a Beckmann thermometer.

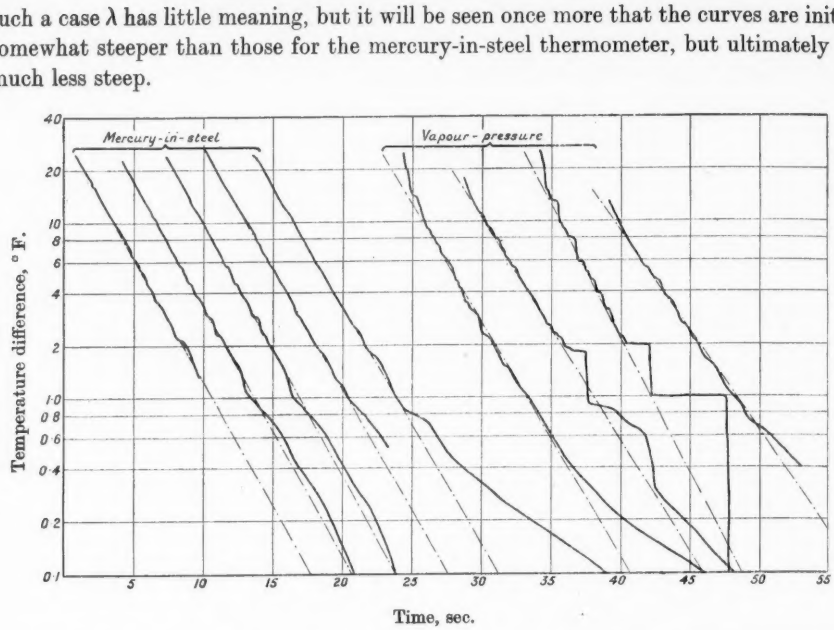
The vapour-pressure thermometer also shows much more evidence of pen friction (horizontal portions of the curve followed by sudden drops) than does the other.



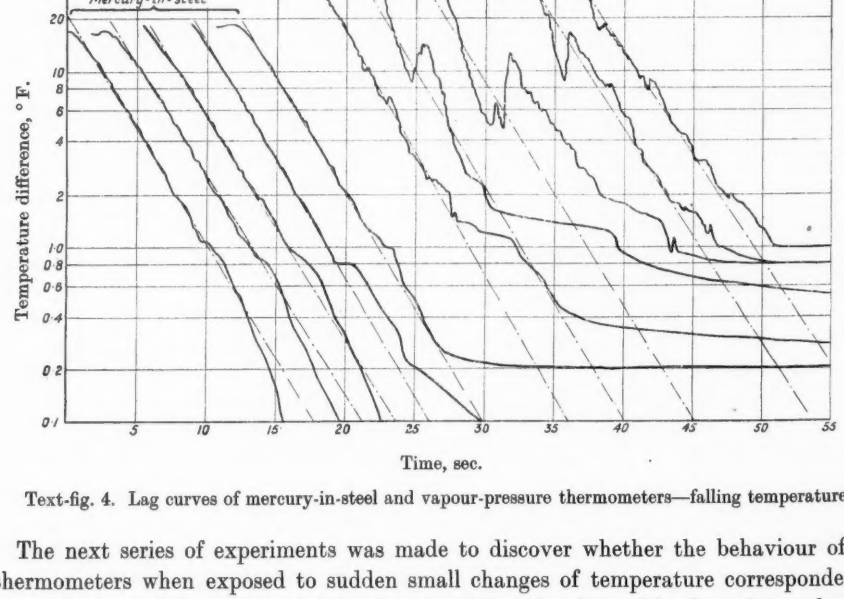
Text-fig. 2. Water-bath used for calibration and lag experiments.

Text-fig. 4 gives corresponding results for falling readings. Several of the curves include a more or less horizontal portion at the start, the record having been begun before the bulb was plunged into the bath. In this way a complete record from start to finish of the temperature change is obtained. The curves for the mercury-in-steel recorder are not markedly different from those obtained on a rising reading, but the inclusion of the initial part of the curve shows that the beginning is less steep than the main portion, an effect opposite to that experienced with the vapour-pressure thermometer. In one of the series of observations, the temperature difference was not reduced to 0·1° F. until after the lapse of some 90 sec. This effect (presumably of pen friction) is again much more marked in the curves for the vapour-pressure recorder, with which the times taken to reduce the difference to 0·1° F. were 110, 160, 170, 230 and 300 sec. With this instrument the curves exhibit further anomalies, all showing (in a more or less marked fashion) a reverse movement of the pen-arm at one or more stages. Clearly, in

such a case λ has little meaning, but it will be seen once more that the curves are initially somewhat steeper than those for the mercury-in-steel thermometer, but ultimately very much less steep.



Text-fig. 3. Lag curves of mercury-in-steel and vapour-pressure thermometers—rising temperatures.



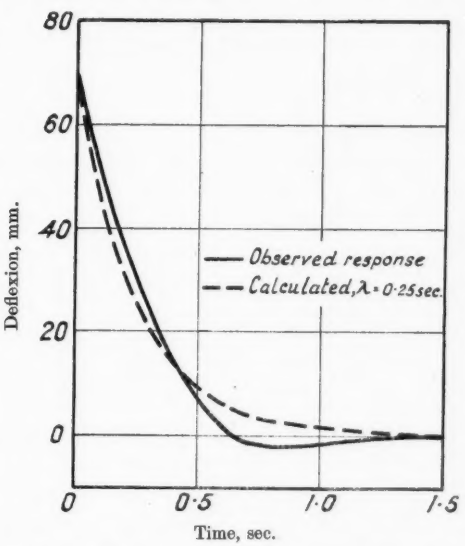
Text-fig. 4. Lag curves of mercury-in-steel and vapour-pressure thermometers—falling temperatures.

The next series of experiments was made to discover whether the behaviour of the thermometers when exposed to sudden small changes of temperature corresponded to that which would be expected from the experimental values of λ. In order to do this, the same water-bath was set to a steady temperature in the region of 160° F. The bulb

of the thermometer under examination was allowed to come into equilibrium with the temperature of the bath, and a bare copper-constantan thermocouple was placed alongside it at a distance of 3 or 4 cm. to act as a standard of comparison. This couple was made from 26 s.w.g. wires, the copper being enamelled. The thermocouple was connected to a potentiometer using a quick-period Moll galvanometer. The galvanometer spot was focused on to a drum camera having a chart width of 12 cm. and a speed of approximately 1 cm./sec. The potentiometer was adjusted so that if the temperature of the thermocouple fell the spot would be deflected across the paper.

The co-ordinates of the trace were marked as follows: *Time:* A small synchronous motor, running at a speed of 300 r.p.m. and carrying a narrow rotating arm was placed in front of the galvanometer. Breaks in the trace at intervals of 0·2 sec. were thus recorded. *Temperature:* The thermocouple used gave an e.m.f. of 26μV. per °F. at 160° F. Before each experiment the shutter of the camera was momentarily opened twice, using two settings of the potentiometer differing by 26μV., the thermocouple being held at a steady temperature.

The lag of the thermocouple system was measured by plunging the junction into the water-bath, when the trace shown as a full line in Text-fig. 5 was obtained. The galvanometer was evidently slightly under-damped, but the curve does not differ very greatly from that shown as a broken line, which is the calculated curve for $\lambda = 0{\cdot}25$ sec.



Text-fig. 5. Lag curve of Moll galvanometer and 26 s.w.g. thermocouple.

The drum-camera record and the cinematograph film of the pen of the recorder were synchronized as follows. The galvanometer lamp was a 12 V. 48 W. bulb, operated through a resistance on the 110 V. mains and controlled by the same switch as the 110 V. 500 W. lamp which supplied the light for the cinematograph camera. The total time of each run is given by the 0·2 sec. marks on the drum-camera record. Since the movements of the pen-arm were to be slight, the field of view of the cinematograph camera was made as small as possible (Pl. 1, fig. 2), and it was therefore not possible to include a stop-watch, but as the camera motor could be relied upon to keep a constant speed to an accuracy of about 1%, the time interval between frames could be assumed constant and determined by counting the number of frames exposed. Owing to the fact that the galvanometer lamp (though of about the same current rating as the 500 W. lamp) was started through a resistance, its brightness built up more slowly than that of the 500 W. lamp, and it was also found to be extinguished more slowly when switched off. The necessary corrections to correlate the time intervals of the two records were determined by making a special run in which two identical lamps (controlled by the

same switch as the main illuminants) were arranged to form images on the two records. These lamps proved to have similar time characteristics to the 500 W. lamp, but the record of the galvanometer lamp was found to start and finish 0·6 and 0·3 sec. late respectively.

The method used for the examination of the projected image of frames such as that shown in Pl. 1, fig. 2 was to cut a cardboard template to fit the image of the pen and to make a mark on it at the tip. This mark was used in conjunction with a cardboard scale of tenths laid across the chart graduations. In this way, differences of as little as 0·02° F. in the position of the pen-arm could easily be observed.

A representative selection of the results obtained is shown in Text-fig. 6. Two types of experimental procedure were used. The first was to induce a sudden, small drop in temperature of the bath by adding a quantity of cold water through the funnel. The other technique was to use two additions of equal volumes of water, the first at a temperature of about 110° F. and the second boiling. The bath temperature was lowered slightly by the first addition, and was then approximately restored by the boiling water a few seconds later. The volumes of water used varied from 50 to 550 ml. according to the temperature drop required.

In Text-fig. 6 the thermocouple records are shown as full lines, and may be taken as corresponding closely to the actual fluctuations in the bath temperature. The figures in circles used to identify the various curves are (i) the approximate temperature change in °F. and (ii) the interval, in seconds, between the two additions of water. The dotted lines show the response of the instrument under test, while the broken lines give the theoretical response to be expected from a thermometer having a lag constant 3 sec. greater than that of the thermocouple (i.e. about 3·2 sec.).

The calculated curves have been obtained by splitting up the full-line curves into sections. For example, the record of T in the mercury-in-steel curve (0·5/—) was taken to be (i) a straight line falling from 0 to $-0{\cdot}5°$ F. between $t=0$ and $t=0{\cdot}4$ sec., (ii) a sine curve of period 3 sec. and semi-amplitude 0·8° F. between $t=0{\cdot}4$ and $t=1{\cdot}9$ sec., (iii) a sine curve of period 3 sec. and semi-amplitude 0·22° F. between $t=1{\cdot}9$ and $t=3{\cdot}4$ sec., and (iv) constant at $-0{\cdot}5°$ F. from 3·4 sec. onwards.

The appropriate equations are, for the straight line,

$$R = a\lambda(1-e^{-t/\lambda})-at, \tag{iii}$$

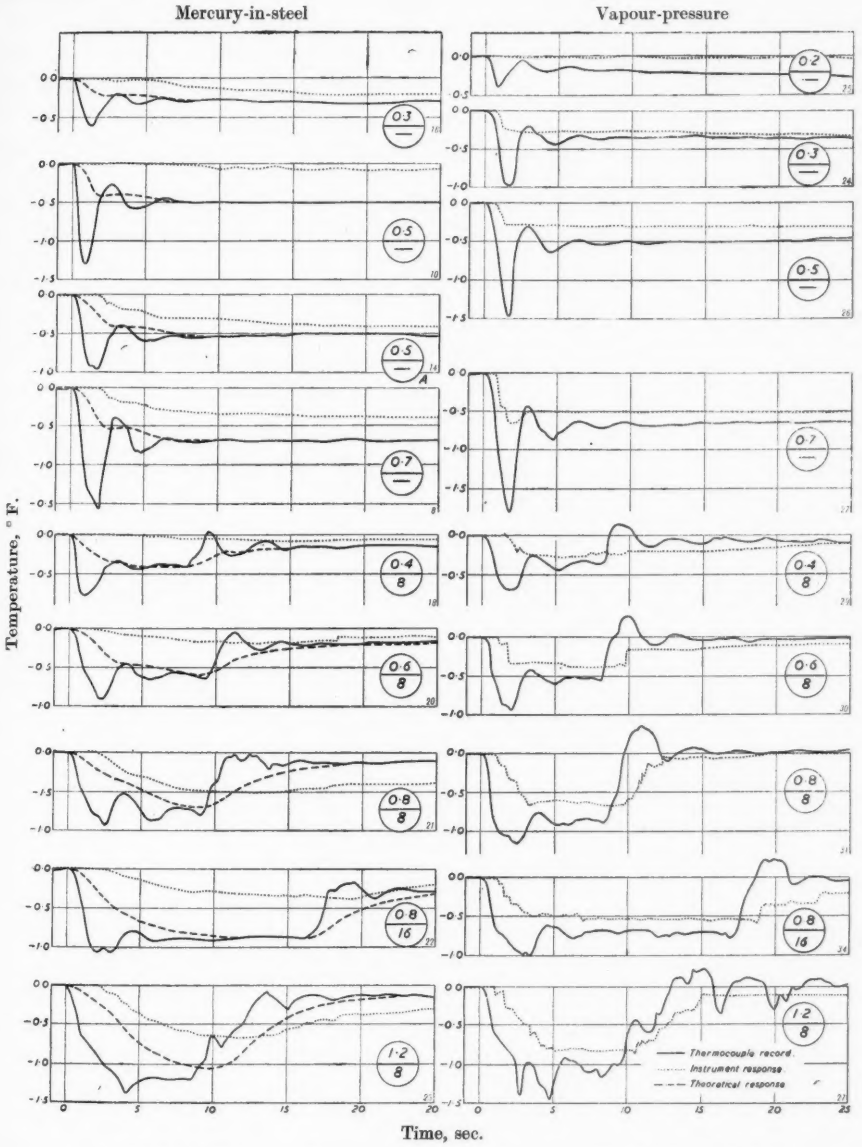
the temperature varying according to the law $T=T_1-at$, and for the sine curve,

$$R = Ce^{-t/\lambda}+T_1+\frac{T_2}{\lambda}\frac{\left(\frac{\sin pt}{\lambda}-p\cos pt\right)}{p^2+\frac{1}{\lambda^2}}, \tag{iv}$$

the temperature varying according to the law $T=T_1+T_2 \sin pt$. The value of C is chosen to give an initial value of R equal to that calculated for the end of the section immediately preceding.

It will be seen that in three of the curves obtained with the mercury-in-steel thermometer, (0·5/—), (0·4/8) and (0·6/8), practically no response has been made to the temperature change. In (0·8/16) the dip has been virtually 'short-circuited', while in none of the other curves is the theoretical response approached. Moreover, the first movement

of the pointer never takes place until about 2 sec. have elapsed after the initial change in temperature.



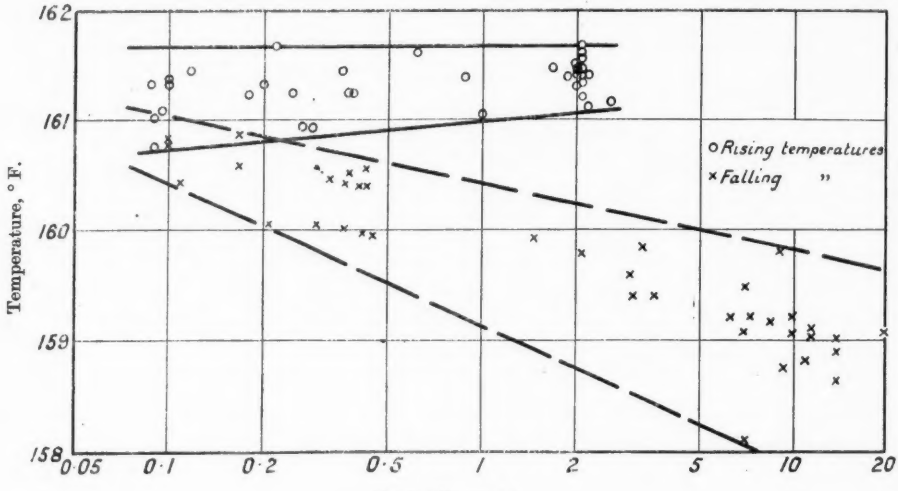
Text-fig. 6. Response of mercury-in-steel and vapour-pressure thermometers to small changes of temperature.

The curves obtained with the vapour-pressure instrument are more satisfactory. Here, the initial delay is reduced to about 1 sec. and in only one case (0·2/—) is there a complete failure to respond, the normal response being about 75% of the temperature change

experienced. There is evidence of pen friction in the form of the curve for (0·6/8). It will be seen that the initial large dip in temperature which normally persists over the first three seconds of the record is completely ignored except in the case of (0·7/—), where its influence can just be detected. On the whole, however, apart from the initial delay of about 1 sec., the curves do not compare badly with the 3 sec. theoretical curves. No attempt was made to calculate theoretical curves for the lag characteristics shown by the vapour-pressure thermometer.

(iii) *Behaviour of control contacts*

The control contacts fitted to the mercury-in-steel recorder are mechanically operated by the movement of the pen-arm. As the temperature rises, the arm reaches one of the contacts and opens the circuit by lifting it against a spring. The flow-diversion valve is thus operated on a falling temperature by the closing of the contacts. This system is



Text-fig. 7. Operation of contacts: mercury-in-steel recorder A.

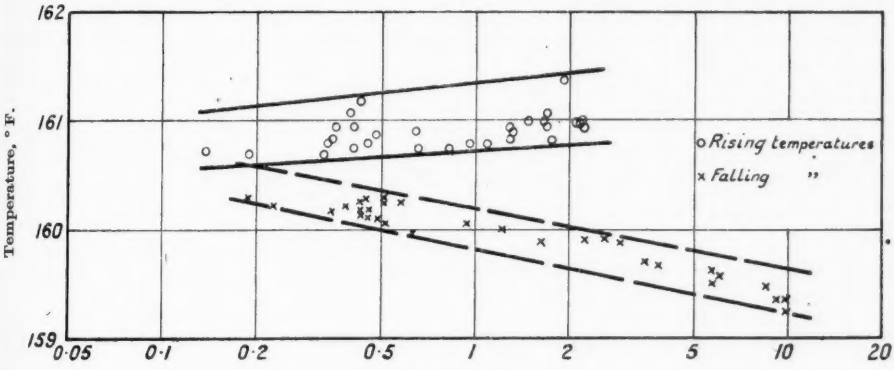
clearly open to objection, as a failure of the electrical supply would cause the valve to remain permanently in the undiverted position.

In order to investigate the operation of the contacts, they were connected in series with an indicator and a small choke-coil passing about 7 ma. on the 230 V. a.c. mains. This circuit simulates the normal conditions of use. The thermometer bulb was placed in the water-bath and the temperature was measured by the same thermocouple system as had been used for the lag experiments. The operating temperature of the contacts was observed at a variety of rates of rise and fall of temperature. The maximum rate of rise obtainable was just over 2° F. per min., but, by running a stream of cold water into the bath, the rate of fall could be made as much as 15 or 20° F. per min., which was about as fast as it could be followed on the potentiometer.

Text-fig. 7 gives a plot of all the results obtained with recorder A. It seems probable, from the observations on falling temperatures, that the poor results were mainly caused

by pen friction which made itself more and more apparent as the pen endeavoured to follow the more rapid changes of temperature. When these results were discussed with the makers of the instrument, they suggested trying a recorder in which the pen-arm had been loaded so as to aid its return on a falling temperature. Recorder B was so treated and gave the results plotted in Text-fig. 8, which, while better than those in Text-fig. 7, are still far from ideal.

Table 2 summarizes the observations. In these experiments and the corresponding ones on the vapour-pressure indicator which follow, the control contact was set to 160° F. on the scale of the instrument. The figure in brackets at the head of each column of the table is the temperature at which the contact might have been expected to operate when the calibration corrections given in Table 1 are taken into account.



Text-fig. 8. Operation of contacts: mercury-in-steel recorder B.

Table 2. *Contact operating temperatures—mercury-in-steel recorders*

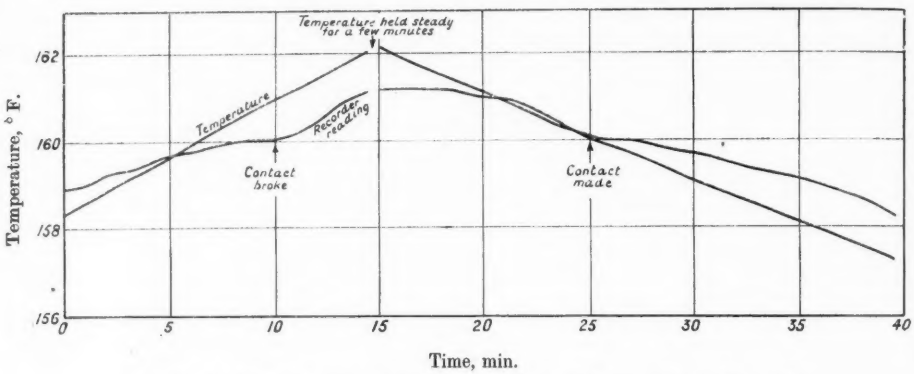
Rate of rise or fall °F. per min.	Recorder A		Recorder B	
	Rising (160·8) °F.	Falling (159·7) °F.	Rising (160·3) °F.	Falling (159·4) °F.
0·1	161·3±0·5	160·7±0·3	160·8±0·3	160·6±0·2
2	161·4±0·3	159·5±0·7	161·0±0·3	160·0±0·2
10	—	158·9±0·9	—	159·5±0·2

At the higher rates of fall the uncertainty in the operating temperature is much less with recorder B than with recorder A, while the change in the operating temperature between rates of 0·1 and 10° F. per min. is reduced from 1·8 to 1·1° F. Making allowance for a lag constant of 3 sec., one would expect the contact to operate about 0·5° F. lower at a rate of fall of 10° F. per min. than at a very slow rate, so that the figure of 1·1° F. is still very high, and suggests that the operating mechanism of the contacts should be separated entirely from the recording pen, so that if the latter is held up by friction on a rapidly falling temperature, the operation of the flow-diversion valve will not be affected.

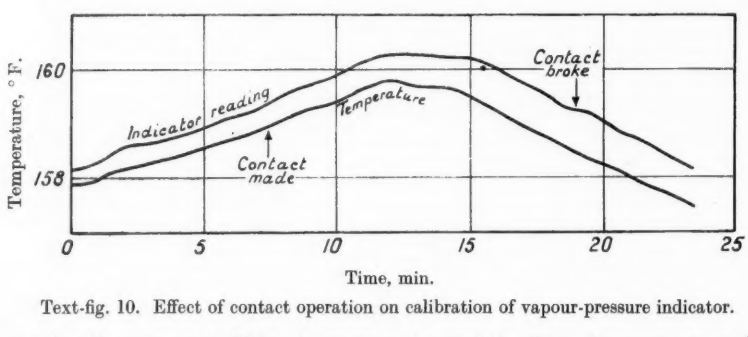
A further point to be investigated in connexion with the operation of the contacts was to find whether the accuracy of the calibration was appreciably affected by the extra resistance offered to a rising movement of the pen when it was holding the contacts

open (i.e. at temperatures above the control temperature). To this end, the readings of the recorder were compared with those of a standard mercury-in-glass thermometer in the water-bath on both rising and falling readings past the contact point (160° F.). The results are shown in Text-fig. 9. The corrections obtained on a rising reading just below and just above the range of readings in which the contact is being lifted (159·3–160·5° F.) are −0·4 and +1·0° F. respectively, while on a falling temperature the corresponding figures are −1·0 and −0·05° F. The lifting of the contact, then, lowers the reading of the recorder by 1° F. or more.

In the vapour-pressure indicator, pneumatic operation of the contacts is used with a view to avoiding any effect on the calibration. Air is led into the instrument at a



Text-fig. 9. Effect of contact operation on calibration of mercury-in-steel recorder.



Text-fig. 10. Effect of contact operation on calibration of vapour-pressure indicator.

pressure of 15 lb. per sq.in. This air pressure is used to close the contacts and thus to energize an electrical relay which then holds the flow-diversion valve in the undiverted position. When the temperature is below the control point, a jet in the air-pressure line allows air to leak away so that the contacts are not closed. No current then passes through the relay and the flow is diverted. If the temperature rises above the operating point, a vane attached to the pen-arm passes over the jet and reduces the leak; the pressure builds up and closes the contacts so that the valve can then be set in the undiverted position. It will be noted that if either the compressed air or the electrical supply fails, the valve should automatically take up the diverted position.

With this system it was found that the operation of the contacts had a negligible effect on the calibration of the indicator. Text-fig. 10 gives results corresponding to those of

Text-fig. 9 for the mechanically-operated contacts. These two graphs show clearly the difference between the values of the corrections for rising and falling readings.

Experiments similar to those recorded in Text-figs. 7 and 8 were made at working pressures of 15, 13½ and 10 lb. per sq.in. in order to investigate, in addition, the effect of variations in pressure on the operation of the contacts. The graphs of Text-figs. 11–13 show the results, which are also summarized in Table 3.

Table 3. *Contact-operating temperatures—vapour-pressure indicator*

	Air pressure					
	15 lb. per sq.in.		13½ lb. per sq.in.		10 lb. per sq.in.	
Rate of rise or fall °F. per min.	Rising (159·5) °F.	Falling (159·0) °F.	Rising (159·5) °F.	Falling (159·0) °F.	Rising (159·5) °F.	Falling (159·0) °F.
0·1	159·1±0·3	159·1±0·4	158·7±0·2	158·5±0·1	158·3±0·1	158·0±0·2
2	159·1±0·3	158·5±0·4	159·0±0·2	158·2±0·2	158·5±0·3	157·7±0·2
10	—	157·8±0·3	—	158·0±0·2	—	157·6±0·2

Although 15 lb. per sq.in. is the recommended operating pressure, considerably more consistent results were obtained on falling temperatures by working at a somewhat lower pressure, the dependence of the operating temperature on the rate of fall being reduced from over 1°F. to about 0·5°F., i.e. to the value which would be expected for a lag constant of 3 sec.

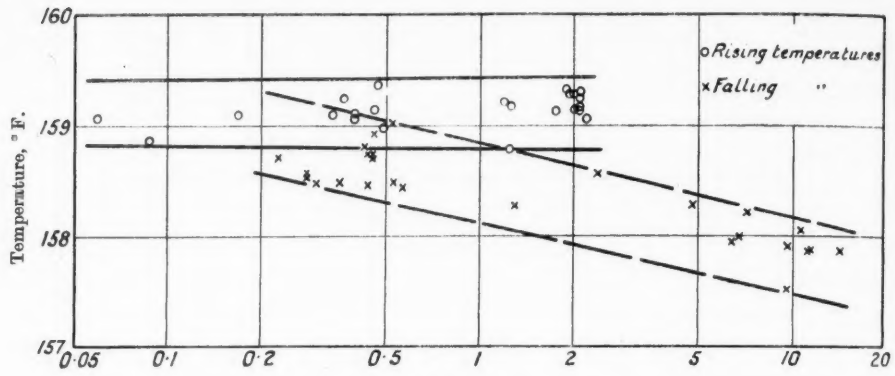
In comparing these results with those obtained for the mercury-in-steel thermometer, it must be remembered that the vapour-pressure instrument was an indicator instead of a recorder, so that it would be reasonable to expect better results owing to the absence of pen friction. The effect of rate of rise or fall of temperature is dependent on the time taken for the air pressure to adjust itself from zero or the full working pressure to the pressure at which the contacts close or open. This pressure was found to be 4·6, 4·0 or 3·2 lb. per sq.in. according to whether the line pressure was 15, 13½ or 10 lb. per sq.in. The results obtained suggest that the pressure range should be kept as small as possible consistent with reliability of operation.

The difference between the 'rising' and 'falling' operating temperatures is not widely different from what would be expected from the calibration given in Table 1, though the actual temperatures are lower than would have been expected. The discrepancies are covered by a calibration uncertainty of ±1°F., but it is clear that the operating temperature is not independent of the air pressure except for rapid rates of fall.

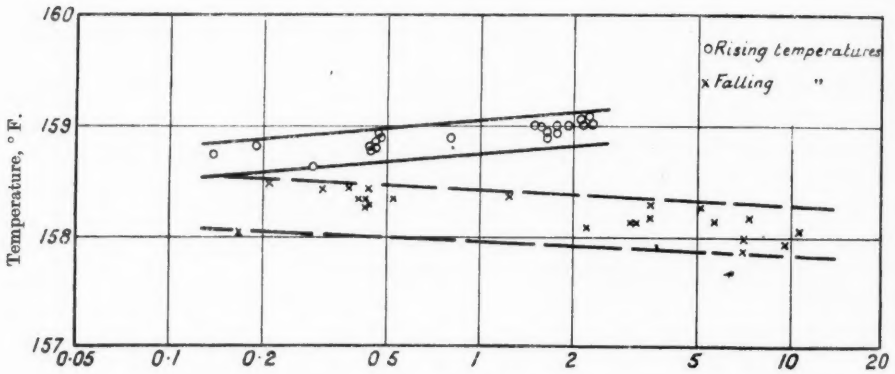
B. THERMOCOUPLE METHOD

Since the results obtained in section A led to the conclusion that something better than the usual type of recording thermometer was desirable, both on the score of accuracy and of quickness of response, experiments were made to investigate whether a thermocouple used in conjunction with recording and controlling gear of a robust type was likely to be satisfactory.

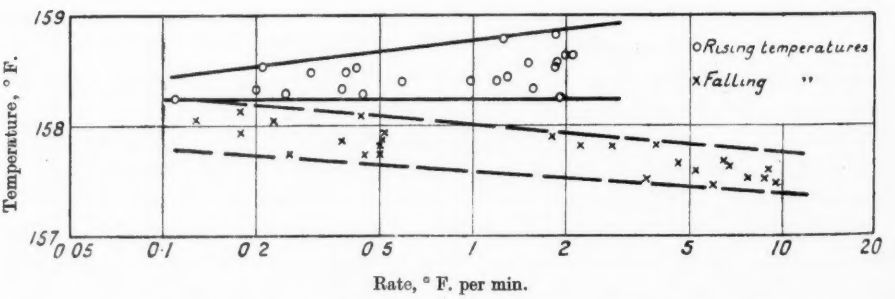
Thermocouples of fine wire used with a special quick-period galvanometer had already proved satisfactory in work carried out at the National Institute for Research in Dairying to investigate the temperature distribution in the milk passing through an H.T.S.T. plant, and a high-speed recorder of robust character was known to be available. This instru-



Text-fig. 11. Operation of contacts: vapour-pressure indicator, 15 lb. per sq.in.



Text-fig. 12. Operation of contacts: vapour-pressure indicator, 13½ lb. per sq.in.



Text-fig. 13. Operation of contacts: vapour-pressure indicator, 10 lb. per sq.in.

ment(6) had proved itself very useful in taking rapid temperature measurements of molten steel under the exacting conditions of steel-works practice, and it was decided to experiment with an instrument of this type for the present investigation. The d.c. amplifier used in this apparatus is essentially a self-balancing potentiometer. The potentiometer comprises a standard resistance (r), across which the unknown e.m.f. is applied, in series with a thyratron valve. In the grid circuit of the thyratron is a photo-electric cell. When the galvanometer in the potentiometer circuit (a robust, taut-suspension, quick-period instrument) is deflected by the application of an e.m.f. across r, light from its mirror falls on the photo-cell and this causes current to flow through the thyratron. 'The effective current output of the thyratron is governed by the degree of illumination of the photo-cell, or by the deflexion of the galvanometer. This output is fed back through the resistance r in such a direction that the voltage drop opposes the applied voltage. Thus when an input voltage causes the galvanometer to deflect, the voltage drop on the resistance r rises until the input voltage is balanced. The output current is therefore proportional to the input voltage since it is the output current which produces the opposing voltage drop, which is held in equilibrium by the galvanometer'(6). An ammeter in the anode circuit of the thyratron thus gives a direct measurement of the input voltage. In the outfit used in the present work r was 0·01 ohm, so that for inputs up to 450μV., the range of the ammeter would be 45 ma.

Any number of current-measuring instruments, relays, etc., may be placed in the circuit, and as the power available is about 2 W. the movements can be substantial and strongly controlled. Moreover, all the electrical gear may be placed at any distance from the plant, with, say, one indicator or recorder within sight of the operator.

The recorder used in the present work had a circular chart with a full-scale width of 4 in. which corresponded to 10°F. if two chromel-alumel couples were used in series. Fortunately, these two alloys, which have been developed and standardized for thermocouple work, are of such composition (see Table 4) as to be likely to be harmless when used bare in milk. It has also been established that satisfactory readings can be obtained when the couples are used in this way.

Table 4. *Composition of chromel and alumel wires*

	Chromel (%)	Alumel (%)		Chromel (%)	Alumel (%)
Ni	89·0	94·0	Si	—	1·0
Cr	9·8	—	Fe	1·0	0·5
Al	—	2·0	Mn	0·2	2·5

(i) *Accuracy*

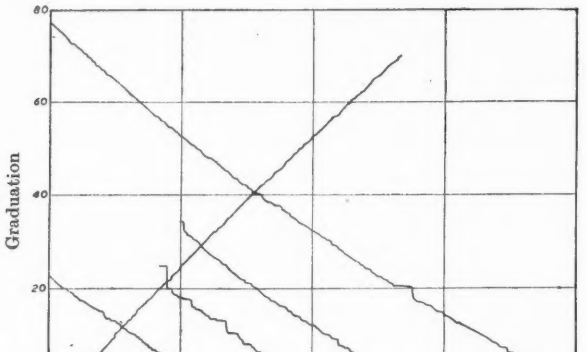
The thermocouple is a device which measures the difference in temperature between two points: the 'hot junction' and the 'cold junction'. The 'hot junction' is placed at the point where the temperature is to be measured and the 'cold junction' is maintained at a known, fixed temperature. On occasion, the 'cold junction' temperature may actually be higher than that of the 'hot junction'.

In order that variations in e.m.f. between different couples of the same nominal characteristics (which may amount to 1% or so) may be negligible, it is desirable to keep the difference in temperature between the hot and cold junctions as small as possible. If, for example, the cold junction is kept in ice, a 1% variation in e.m.f. would give rise

to an error of 1·3° F at 160° F., but if the cold junction were at 150 or 170° F., this error would be reduced to 0·1° F. and could safely be neglected.

If, then, the difference between hot- and cold-junction temperatures is kept small, the overall accuracy depends on two factors only: (i) the accuracy with which the cold-junction temperature can be maintained and (ii) the accuracy of calibration of the measuring instrument.

As regards the first of these, the controlled hot-water supply to the plant affords a fairly steady temperature just above 160° F., and could probably be used for the cold junctions. This would not be a satisfactory arrangement without further precautions, since the water and milk temperatures would tend to rise and fall together and the temperature changes would thus not be indicated. It is, however, possible to compensate for variations in cold-junction temperature by automatic means. If, for example, such a device reduces the effect of a change in cold-junction temperature to 2% of its normal



Text-fig. 14. Response of 'd.c. amplifier' thermocouple recorder to steady changes of temperature.

value, a fall in temperature of 5° F. in the water temperature would only introduce an error of 0·1° F.

As to the constancy of calibration of the amplifier-recorder system, a series of calibrations carried out over a period of months, during which time the amplifier was readjusted more than once for experimental purposes, showed an overall variation of just over ±1%, or ±0·1° F. at full-scale deflexion. Moreover, means can be provided (7) whereby the accuracy of the calibration can be checked by a simple push-button operation.

The observations plotted in Text-fig. 14 were made in order to discover whether the instrument was as liable to 'stiction' effects on a falling temperature as the other recorders had been. The thermocouple was placed in a water-bath set so as to give a slowly rising or falling temperature and readings were taken by means of the cinematograph camera at intervals of about 3 sec. The rising and falling curves are similar in their upper portions, but in the lower part of the scale there is evidence of stiction on a falling reading: at its worst this amounted to about 0·4° F. at about graduation 20 of the 100-graduation scale of the chart (10 graduations = 1° F.). As has been mentioned, the

cold junction can be arranged to be at a higher temperature than the hot junction, so that rising readings are then obtained on a falling temperature. In this way, maximum certainty of operation of the diversion valve would be obtained, while for rising temperatures (falling deflexions) it would be desirable to avoid the bottom 25% of the scale. Apart from this 'stiction' effect (which might be reduced by the use of a different recorder unit) no difference could be detected between readings taken on rising and falling temperatures.

Evidently, then, the problem of accuracy in thermocouple measurement in a pasteurization plant reduces itself to that of the close control of cold-junction temperature, followed by automatic compensation to reduce the effect of residual fluctuations to about $\pm 0{\cdot}1°$F. If this can be done, then the overall accuracy of the temperature measurement should be within $\pm 0{\cdot}3°$F., provided that the measuring instruments are calibrated to $\pm 1\%$ of full scale—a quite normal commercial standard of accuracy.

(ii) *Thermometric lag*

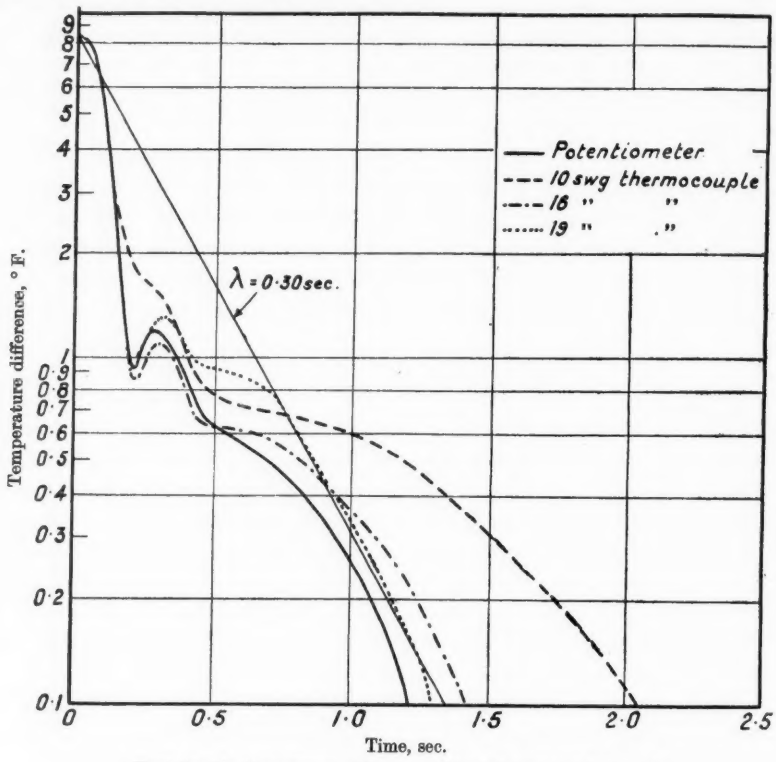
The lag of a thermocouple indicator or recorder is made up of two components: one is the lag of the hot junction of the couple in reaching the desired temperature, and the other depends on the period and damping (and hence the quickness of response) of the galvanometer. An analysis of the lag of the 'd.c. amplifier' recorder was made by means of two experiments. In the first an e.m.f. was applied suddenly and the response was recorded with the aid of the cinematograph camera. For the second, thermocouples of different gauges of wire were connected to the recorder in turn and the hot junctions were plunged into the stirred water-bath. In all these experiments the conditions were such as to give a rising reading on the recorder and, in view of the high speed of response, the camera was run at 64 frames per second.

The results are plotted in Text-fig. 15. The curves obtained depart considerably from the straight-line form, but the full-line curve giving the response of the recorder alone shows that its lag is somewhat less than 0·3 sec. when the full length of the curve is taken into consideration and very much less in the initial portion. It is also clear that the use of a thermocouple of 16 s.w.g. or finer does not appreciably add to the lag, but that a 10 s.w.g. couple increases it by about 75%.

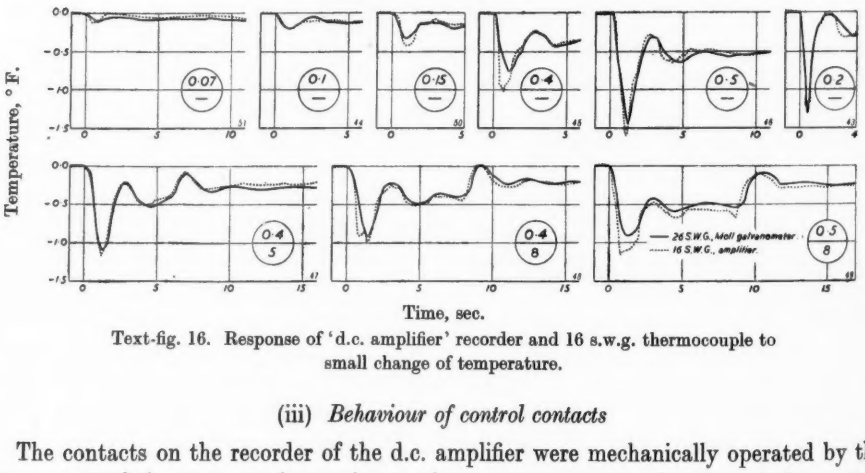
The lag of this equipment is therefore not very different from that of the Moll galvanometer used as a standard in the experiments of which the results are plotted in Text-fig. 6. Experiments similar to these were carried out with the d.c. amplifier and its recorder using a 16 s.w.g. double chromel-alumel thermocouple, and the results are given in Text-fig. 16. The correspondence with the curves given by the Moll galvanometer is very close; in most instances the d.c. amplifier shows a slightly better response than the standard ($\lambda = 0{\cdot}25$ sec.). In one experiment (0·2/—) the Moll record shows a slightly bigger response to the initial swing of the curve, but this experiment was abnormal in that the cold water was added directly through the open top of the bath instead of through the funnel. There is therefore much more possibility that, through imperfect mixing, the two thermocouples did not receive similar temperature impulses. The experiment is of value, however, as it gives some idea of the response to a much more sudden change of temperature than could have been realized by the normal technique.

While transient impulses of this kind can be satisfactorily recorded by photographing the pen, the ink supply in the present instrument was not sufficient to mark the chart

at such a high rate of pen movement. If a recorder of this type is to be used, therefore, the design of the pen needs improvement.



Text-fig. 15. Lag curves of 'd.c. amplifier' thermocouple recorder.



Text-fig. 16. Response of 'd.c. amplifier' recorder and 16 s.w.g. thermocouple to small change of temperature.

(iii) *Behaviour of control contacts*

The contacts on the recorder of the d.c. amplifier were mechanically operated by the movement of the pen-arm, but owing to the greater openness of scale and the greater power available for moving the pen-arm, better operation than was experienced with

the mercury-in-steel recorder might be expected. The contact was set to operate at 160° F., and a series of experiments similar to those made with the other instruments (Text-figs. 6, 7, 10, 11 and 12) was carried out. No difference could be detected between the operating temperatures on rising and falling readings. The rates of rise varied from 0·14 to 2·1° F. per min., and the rates of fall from 0·05 to 3·2° F. per min., while the temperature measurement was sensitive to 0·04° F. Six rising and seven falling operations were observed and none lay outside the range 160·00–160·07° F.

The curves of Text-fig. 13 show that the operation of the contacts has a negligible effect on the calibration of the recorder. The contacts were set to operate at graduation 40 and there is no evidence of any halt in the rising curve at this point, while the slight halt in the falling curve is no greater than the one at graduation 38 which is presumably solely due to the effect of pen friction.

CONCLUSIONS

Table 5 shows a rough comparison of the performances of the three types of instrument which have been studied. The various causes of uncertainty which are listed in this table are additive. For example, considering the mercury-in-steel recorder, the use of a 'rising' calibration on a falling temperature might lead to an error of 2° F. when the possible uncertainty in the calibration is taken into account. With the thermocouple outfit, however, the errors due to other causes than calibration uncertainty are negligibly small. Moreover, the calibration uncertainty of ±0·3° F. is itself made up of three independent sources of error, each of about ±0·1° F., and would therefore not be experienced unless the errors of cold-junction compensation and departure from calibration of both the thermocouple and the recorder were all at a maximum and of the same sign.

Table 5. *Comparison of errors of different types of recorder*

Cause of uncertainty	Magnitude of error: Mercury °F.	Vapour-pressure °F.	Thermo-couple °F.
Calibration	±1	±1	±0·3
Difference between rising and falling calibrations	1	1	0·00
'Stiction': Rising reading	−0·2	−1	−0·04
Falling reading	+0·2	+1	+0·4*
Effect of contact operation	−1	0	0·00
Variation in contact operating temperature	±1†	±0·5‡	±0·04
Approximate lag constant	3 sec.	2·5 sec.	0·2 sec.
Response to sudden small drop in temperature (percentage of drop recorded)	10 to 60%	About 75%	100%
Initial delay in response to sudden drop in temperature	About 2 sec.§	About 1 sec.	Nil

* Lower end of scale only.
† Recorder B.
‡ Indicator operated at 15 lb. per sq.in.: uncertainty reduced to ±0·3° F. at lower working pressures.
§ Except in those cases when the pen failed completely to respond.

Even with the use of the d.c. amplifier it would probably be desirable to separate the operation of the valve from that of the recording pen, but the extra power available removes the objection to direct mechanical operation of the contacts. In consequence, an electrical impulse can be transmitted directly to the flow-diversion valve and the lag caused, for example, by the need for establishing a change in air pressure is eliminated. Any lag additional to that of the recorder is then limited to that of the movement of the valve itself. The use of a valve worked by a strong spring should reduce this to a very small amount.

It seems clear, from the results summarized above, that the use of thermocouples for measuring the temperatures in H.T.S.T. pasteurization can be made to offer considerable advantages over the methods commonly employed, for both accuracy and quickness of response. As regards the latter property the thermocouple is almost ideal, while there seems no reason to doubt that the accuracy of temperature measurement and control could be made about four times as great as is now attained in practice.

The need to operate a pasteurization plant within the narrowest possible limits of temperature has already been pointed out, and the improved accuracy and speed of control made possible by the use of thermocouples would effect a corresponding reduction in the margin of safety which has to be allowed between the minimum safe temperature and that at which the machine is set to operate.

Summary

An investigation has been made into the accuracy and speed of response of temperature-measuring instruments suitable for use in the control of H.T.S.T. pasteurization. Thermocouples used in conjunction with a 'd.c. amplifier' recorder system are shown to be markedly superior to both vapour-pressure and mercury-in-steel thermometers. The accuracy of temperature measurement is about $\pm 0{\cdot}3°$F. as against $\pm 1°$F., while the lag constant is about 0·2 sec. as against about 3 sec. Response to small, rapid and transient changes of temperature is also shown to be greatly improved. The operation of control contacts by the thermocouple recorder was found to be reliable to well within the limit of accuracy of calibration, but the other types showed variations of the order of $\pm 1°$F., in addition to the uncertainties of calibration.

The work described in this paper was undertaken at the request of the National Institute for Research in Dairying as part of the research programme of the National Physical Laboratory, and is published by permission of the Director of the Laboratory. The author wishes to acknowledge the help he has received from Dr A. T. R. Mattick and Miss E. R. Hiscox of the National Institute for Research in Dairying and from his colleagues Mr C. Wigley and Miss V. M. Leaver. Dr Mattick and Miss Hiscox supplied the experimental results given in the introduction, for the drafting of which they are largely responsible. Mr Wigley derived the equations used in computing the theoretical curves of Text-fig. 6 and Miss Leaver assisted with the observational work.

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317. THE RESAZURIN AND METHYLENE-BLUE TESTS AS A MEASURE OF THE KEEPING QUALITY OF MILK

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Introduction

During recent years an extensive literature on the reduction of resazurin has appeared, particularly in comparison with methylene blue, as a measure of the bacteriological quality of milk. Little information, however, is available on its value for measuring the keeping quality (K.Q.) as would be judged by the consumer, that is to say, by the senses of taste and smell.

Wilson and his colleagues(1) obtained a mean figure of +0·437 for the correlation coefficient (r) of methylene-blue reduction time at 37° C. and K.Q. at 17·5° C., using smell as a criterion of K.Q., and of +0·475 for the correlation coefficient of methylene-blue reduction time at 17·5° C. and K.Q. at 17·5° C.; but to our knowledge no comparable figures exist for resazurin.

Moldavan(2) graded milks after 1 hr. incubation at 37° C., using resazurin as the indicator, into five classes, and stated that they agreed well with the K.Q., as tested by smell and taste after incubation for 16 hr. at 70° F. Indirect comparison has been made by Thomas & Davies(3) using the methylene-blue reduction time at 15° C. as a measure of K.Q.; they state that the results of the two tests showed a fairly good correlation. Barkworth and his colleagues(4) reported the results of examining 200–400 samples of milk, using clot on boiling as a criterion of K.Q. The number of milks examined by the methylene-blue and resazurin tests was not the same and the differences were very few, but, according to them, there was a suggestion that the 10 min. resazurin test afforded a better index of K.Q. than the ¼–½ hr. methylene-blue test. Davis, Newland & Knuckey(5), examining 78 samples during 1 month, studied the relationship between the resazurin test and clot on boiling by means of scatter diagrams. For a 10 min. test at 37° C. they state: 'Ignoring the usual few outside points, the correlation is as good as one can hope to get with any two bacteriological tests'; but with the 1 hr. resazurin test at 37° C. the correlation is stated to be not so good.

When, in 1942, the Ministry of Agriculture proposed the use of a resazurin test as a measure of the K.Q. of raw milk, it seemed desirable, in view of the scantiness of the published information, to study the relation between the resazurin test and the K.Q. In deciding which tests to use for estimating the K.Q., we were influenced by our previous experience, which had shown us that the clot-on-boiling test was insufficiently sensitive, and that the sense of smell was less delicate than that of taste. Preliminary observations on pasteurized milk had revealed the value of an alcohol precipitation test; in only a small number of samples was a milk condemned by taste before being precipitated by alcohol. As we had two expert milk tasters in the laboratory, we decided to examine every sample by both the taste and the alcohol tests. It was thought wise to include the standard methylene-blue test as a control, and to examine a sufficient number of samples to permit the calculation of a numerical index of the degree of correlation between the two dye tests and the K.Q. of the milk.

Experimental

After some preliminary work the experiment was planned and carried out as follows:

During each of the four periods Oct.–Nov., Jan.–Feb., Apr.–May and July–Aug., about 600 composite samples of morning and evening milk were taken under sterile

conditions from farmers' milk arriving in churns at the receiving platform, and immediately transferred to the laboratory. Two sterile 10 ml. tubes were filled to the 10 ml. mark, and these and the balance of the sample were placed in a ventilated box on a north wall, where they remained until 2 p.m. Not more than eighteen samples were examined on any one day to allow of ample time for manipulation. At 2 p.m., 1 ml. of each of the standard dye solutions was added to the respective tube, and after one inversion the tubes were placed immediately in a water-bath thermostatically controlled at $37 \pm 0{\cdot}5°$ C.

The 3 oz. sample bottles, in which the milk was used for the K.Q. test, were at the same time placed in a water-bath thermostatically controlled at 16–18° C.

The disk numbers of the tubes containing the resazurin and milk were read at 10 min. (within $\frac{3}{4}$ min.) and 1 hr., and the time taken to reach disk 1 was recorded. The tubes were inverted once every $\frac{1}{2}$ hr. after the 1 hr. reading. It will be noted that the details of the resazurin test did not follow the exact procedure as laid down by the Ministry of Agriculture in respect of atmospheric temperature compensation, which was not published in detail till after the investigation had started.

The methylene-blue test was carried out in accordance with the technique laid down officially by the Ministry of Health (6), the tubes being inverted once every $\frac{1}{2}$ hr.

The alcohol test was carried out by adding 1 ml. of 68% ethyl alcohol to 1 ml. of the incubated milk, and inverting the tube once. If precipitation of casein, i.e. discrete particles, however small, was observed in the thin layer of liquid adhering to the walls of the tube, the test was regarded as positive. The alcohol was prepared by diluting industrial methylated spirit with distilled water to approximately 68%, neutralizing any free acid, and confirming the final concentration by determining the specific gravity, which should be 0·895 at 15·5° C.

The taste was regarded as positive when any abnormal flavour developed. Among 2588 samples in which the milk was condemned on taste, the following flavours were recorded most frequently:

Tallowy	1445
Mealy	392
Acid	308
Putrid	188
Fruity	59
Bitter	53
	2445

In the remaining samples the following flavours were met with: metallic, cheesy, salty, cooked, strong, powdery, earthy, oily, fishy, soda-like, sweet, onion-like, cat-like.

The taste and alcohol tests were applied at 3 p.m., 9 p.m. and 9 a.m. In the last two periods of the work samples were incubated till both tests were positive, but during the first two periods the samples were occasionally discarded after one or other test had become positive. Throughout the investigation, however, samples were condemned on either the alcohol or the taste test alone. The K.Q. was calculated according to the scheme shown in Table 1 to give what we regard as the true life of the milk, as judged from the time of commencement of the test.

As the samples were composite samples of morning and evening milk, and as the test did not start till 2 p.m. on the day of delivery of the milk to the receiving station, a

figure of about 15 hr. should be added to the results given in the following tables if the true life of the milk from the time of production is required.

Table 1. *Calculation of the* K.Q. *test*

Previous reading	Final reading	End-point reached at
Taste – Alcohol –	Taste + Alcohol +	¼ way between the 2 readings
Taste – Alcohol –	Taste + Alcohol –	½ ,, ,,
Taste – Alcohol –	Taste – Alcohol +	¾ ,, ,,
Taste suspicious Alcohol –	Taste + Alcohol +	¼ ,, ,,
Taste suspicious Alcohol –	Taste + Alcohol –	½ ,, ,,
Taste suspicious Alcohol –	Taste suspicious Alcohol +	½ ,, ,,
Taste – Alcohol –	Taste suspicious Alcohol +	¾ ,, ,,

A suspicious reading, followed by a negative reading, was ignored.

RESULTS

Correlation between the dye reduction and the K.Q. *tests*

In analysing the results, the estimated K.Q. times of the milk were checked by two independent sets of workers. Frequency-distribution tables were then prepared. The disk numbers in the 10 min. and 1 hr. resazurin tests, and the time to reach disk 1 in the resazurin test or the time to complete decolorization in the methylene-blue test, were plotted against the K.Q. in hours of each sample of milk. Correlation coefficients were then worked out in the usual way. The results are summarized in Table 2. It will

Table 2. *Relationship between dye-reduction tests and* K.Q. *Correlation coefficients* (*r*)

	Oct.–Nov.	Jan.–Feb.	Apr.–May	July–Aug.	Arithmetic mean (not weighted)
A. 10 min. resazurin and K.Q.	0·31 (716)	0·43 (576)	0·47 (648)	0·58 (648)	**0·45**
B. 1 hr. resazurin and K.Q.	0·50 (716)	0·54 (576)	0·65 (648)	0·67 (648)	**0·59**
C. Time to reach disk 1, resazurin and K.Q.	0·63 (538)	0·64 (576)	0·75 (648)	0·73 (648)	**0·69**
D. Methylene-blue reduction and K.Q.	0·65 (716)	0·65 (576)	0·75 (648)	0·75 (648)	**0·70**

Note 1. Numbers of milks in parentheses.

Note 2. C. Average time of reduction of resazurin to disk 1 to nearest 5 min.:

Oct.–Nov.	4 hr. 35 min.	Apr.–May	4 hr. 5 min.
Jan.–Feb.	5 hr. 0 min.	July–Aug.	2 hr. 30 min.

Note 3. D. Average time of reduction of methylene blue to nearest 5 min.:

Oct.–Nov.	4 hr. 45 min.	Apr.–May	4 hr. 20 min.
Jan.–Feb.	5 hr. 15 min.	July–Aug.	2 hr. 50 min.

be seen that in each method of examination the results were fairly uniform, the lowest degree of correlation between the dye test and the K.Q. test occurring in the colder months of the year and the highest in the warmer months. A comparison of the different tests shows that the 10 min. resazurin test afforded the least reliable index of K.Q., and the 1 hr. resazurin test a rather better index. Between the resazurin test in which the time to reach disk 1 was measured and the methylene-blue test there was practically

nothing to choose; the latter test, however, gave a slightly higher correlation coefficient in three out of the four trial periods. The correlation between the methylene-blue reduction time and the K.Q. of the milk ($r = 0{\cdot}70$) was very much higher than the figure of 0·437 obtained in a previous investigation(1), when the K.Q. of the milk was judged largely by smell instead of, as in the present series of observations, by a combined taste and alcohol test. It is interesting to note that, in comparing C and D in Table 2, the time taken to reach disk 1 in the resazurin test was, on the average, only 15 min. less than that taken for the decolorization of methylene blue.

From this series of observations on raw milks at four different times of the year, it may be concluded that the best index of K.Q. is given by the time taken to reach disk 1 in the resazurin test and the time taken for the decolorization of methylene blue. The saving of time resulting from the use of the resazurin test is so small that it is doubtful whether it is sufficient to offset the perhaps slightly greater value and ease of performance of the methylene-blue test.

Comparison of the 10 *min. resazurin and the* 30 *min. methylene-blue tests*

For the sake of interest, a comparison has been made between the results of the 10 min. resazurin test and the 30 min. methylene-blue test. The results are set out in Table 3. It will be noticed that, with one exception, every milk giving a disk reading

Table 3. *Comparison between* 10 *min. resazurin and* 30 *min. methylene-blue tests*

	10 min. resazurin test				30 min. methylene-blue test			
	No. of milks giving disk reading 0–½ (K.Q.)	No. of these milks reducing methylene blue in 30 min. (K.Q.)	No. of milks giving disk reading 1–3½ (K.Q.)	No. of these milks reducing methylene blue in 30 min. (K.Q.)	No. of milks reducing methylene blue in 30 min. (K.Q.)	No. of these milks giving 10 min. resazurin disk of		
Period						0–½ (K.Q.)	1–3½ (K.Q.)	4–6 (K.Q.)
Oct.–Nov. 1942	18 (3·7)	18 (3·7)	19 (4·0)	13 (3·8)	38 (4·2)	18 (3·7)	13 (3·8)	7 (6·3)
Jan.–Feb. 1943	5 (3·5)	5 (3·5)	11 (5·0)	7 (4·0)	15 (4·0)	5 (3·5)	7 (4·0)	3 (4·7)
Apr.–May 1943	13 (3·8)	13 (3·8)	25 (8·0)	16 (7·4)	34 (6·0)	13 (3·8)	16 (7·4)	5 (7·3)
July–Aug. 1943	67 (4·0)	66 (4·0)	38 (5·3)	26 (4·5)	104 (4·6)	66 (4·0)	26 (4·5)	12 (8·4)
Total	103 (3·9)	102 (3·9)	93 (5·7)	62 (5·0)	191 (4·7)	102 (3·9)	62 (5·0)	27 (7·2)
Percentage	100	99*	100	60	100	53	32	14

K.Q. = Keeping quality in hours and fractions of an hour.

* 80% of the milks giving a disk reading of 0–½ reduced methylene blue within 10 min.

of 0–½ in the 10 min. resazurin test reduced methylene blue within half an hour. A study of the individual protocols, which cannot be reproduced here, shows that 80% of these milks reduced methylene blue within 10 min. On the other hand, of the 191 milks reducing methylene blue within half an hour, only 53% gave a 10 min. resazurin disk reading of 0–½. If it is agreed that, under peace-time conditions, no milk reducing methylene blue within half an hour should be regarded as fit for human consumption, then it is evident that the rejection standard proposed in the Ministry of Agriculture scheme for war-time use is deplorably low and fails to pick out nearly half of the unsatisfactory milks.

Comparison of taste and alcohol precipitation in the K.Q. *test*

In about 60% of the samples examined the milk was condemned simultaneously by the taste and the alcohol test. In the remainder, one test became positive before the other (Table 4). The alcohol test proved consistently more sensitive than change in taste,

Table 4. *Comparison of taste and alcohol precipitation in the* K.Q. *test*

Period	End-point	No. of milks	%	Average K.Q. in hr.
Oct.–Nov. 1942	T before A	84	11·7	21·5
	A before T	145	20·3	15·0
	Both together	487	68·0	21·3
	Total	716	100·0	20·1
Jan.–Feb. 1943	T before A	109	18·9	22·4
	A before T	122	21·2	22·4
	Both together	345	59·9	24·5
	Total	576	100·0	23·7
Apr.–May 1943	T before A	102	15·7	23·6
	A before T	196	30·3	21·6
	Both together	350	54·0	23·3
	Total	648	100·0	22·8
July–Aug. 1943	T before A	72	11·1	12·5
	A before T	196	30·3	15·8
	Both together	380	58·6	17·0
	Total	648	100·0	16·1
Total	T before A	367	14·2	20·6
	A before T	659	25·5	18·6
	Both together	1562	60·3	21·4
	Total	2588	100·0	20·6

T = taste; A = alcohol precipitation.

becoming positive first in 25·5% of the milks as against 14·2% in which the reverse occurred. It seemed possible that the alcohol test might detect some qualitative change in the milk which was not associated with any alteration in flavour. If so, then the average K.Q. of the milks condemned by the alcohol test first might be different from that of milks condemned by the taste first. Table 4 shows that in the first and third periods the average K.Q. of milks in which the alcohol test became positive before a change in taste was 2–6½ hr. less than when the milk was condemned first by taste; but in the second and fourth periods either no difference was observed at all, or the difference was in the opposite direction. On the whole, it looks as if the combined taste and alcohol K.Q. test may prove of considerable value in practice. Even if taste was omitted altogether, because expert milk tasters were not available, the K.Q. of the milk could be gauged fairly accurately by the alcohol test alone in all but 14% of the samples examined.

Relation of K.Q. *to different disk numbers in the* 10 *min. resazurin test*

The average K.Q. of milks giving different disk numbers in the 10 min. resazurin test has been worked out (Table 5). The results are seen to be irregular. There is no appreciable difference in the K.Q. of milks corresponding to disks 0, ½ and 2, but the numbers are not large enough to justify any generalization.

Table 5. *Relation between disk numbers and* K.Q. *in* 10 *min. resazurin test*

Disk no.	No. of milks	K.Q. in hr. and min. Mean	Standard deviation	Standard error of mean
0–½	103	4 hr. 36 min.	1 hr. 3 min.	6 min.
>½–1	—	—	—	—
>1–2	21	4 hr. 30 min.	0	0
>2–3	54	6 hr. 24 min.	3 hr. 53 min.	32 min.
>3–4	78	8 hr. 18 min.	6 hr. 14 min.	42 min.
>4–5	318	16 hr. 30 min.	10 hr. 36 min.	36 min.
>5–6	2014	23 hr. 0 min.	9 hr. 50 min.	13 min.
Total	2588			

Relation of K.Q. *to different disk numbers in the* 1 *hr. resazurin test*

The average K.Q. of milks giving different disk numbers in the 1 hr. resazurin test is recorded in Table 6. Here, again, the results tend to be irregular, there being little appreciable difference in the K.Q. of milks corresponding to disks 1 and 2.

Table 6. *Relation between disk numbers and* K.Q. *in* 1 *hr. resazurin test*

Disk no.	No. of milks	K.Q. in hr. and min. Mean	Standard deviation	Standard error of mean
0–½	362	9 hr. 24 min.	5 hr. 5 min.	16 min.
>½–1	10	12 hr. 0 min.	4 hr. 53 min.	1 hr. 33 min.
>1–2	137	12 hr. 30 min.	6 hr. 26 min.	33 min.
>2–3	164	15 hr. 24 min.	8 hr. 5 min.	38 min.
>3–4	468	20 hr. 48 min.	9 hr. 39 min.	27 min.
>4–5	1101	24 hr. 48 min.	9 hr. 31 min.	17 min.
>5–6	346	26 hr. 54 min.	8 hr. 23 min.	27 min.
Total	2588			

Relation of K.Q. *to different reduction times in the methylene-blue test*

The average K.Q. of milks reducing after different times of incubation is recorded in Table 7. This table shows a much more uniform distribution of milks into the different classes, and a more constant relationship between the reduction time and the K.Q. of the samples than in either of the two resazurin tests. The increase in average K.Q. goes up fairly regularly by about 3 hr. per class. The standard errors of these values are also almost invariably lower than those in the 1 hr. resazurin test.

Table 7. *Relation between time of reduction and* K.Q. *in methylene-blue test*

Time of reduction in hr.	No. of milks	K.Q. in hr. and min. Mean	Standard deviation	Standard error of mean
0–½	191	5 hr. 18 min.	2 hr. 44 min.	12 min.
>½–1	150	9 hr. 0 min.	5 hr. 18 min.	26 min.
>1–2	225	11 hr. 18 min.	5 hr. 40 min.	23 min.
>2–3	230	14 hr. 18 min.	6 hr. 36 min.	26 min.
>3–4	224	17 hr. 30 min.	6 hr. 54 min.	28 min.
>4–5	219	20 hr. 30 min.	7 hr. 15 min.	29 min.
>5–6	290	24 hr. 24 min.	8 hr. 30 min.	30 min.
>6	1059	27 hr. 48 min.	9 hr. 1 min.	17 min.
Total	2588			

SUMMARY AND CONCLUSIONS

1. During four periods equally spaced throughout the year raw milks coming from surrounding farms into the Buckingham receiving station were examined by (*a*) the 10 min. resazurin test, (*b*) the 1 hr. resazurin test, (*c*) the resazurin test in which the time to reach disk 1 was recorded, (*d*) the modified methylene-blue test, and (*e*) a keeping quality test based on taste and alcohol precipitation. Altogether, 2588 samples were examined.

2. Table 2, in which the correlation coefficients are worked out between the results of the dye and the K.Q. tests, shows that there is little to choose between the methylene-blue test and the time to reach disk 1 in the resazurin test; but since an average of only 15 min. was saved by carrying out the resazurin test, there seems little advantage to be gained by the use of this test. Both of these tests are superior to the 10 min. and the 1 hr. resazurin tests.

3. A comparison of Tables 5–7, in which the average K.Q. of the milks corresponding to different disk numbers or reduction times is recorded, shows that the methylene-blue test affords the best indication of the K.Q.

4. A study of Table 3, setting out the relation between the 10 min. resazurin and the methylene-blue reduction tests, shows that 99% of milks giving a 0–½ reading in the resazurin test reduce methylene blue within 30 min. and 80% of them within 10 min.; but that, of milks reducing methylene blue within 30 min., only 53% give a disk reading of 0–½ in the 10 min. resazurin test. If it is agreed that no raw milk on arrival at the creamery which reduces methylene blue within 30 min. and which has, according to Table 7, an average K.Q. of only about 5 hr. at the time of testing, is fit for human consumption, then it is clear that the present rejection standard in the 10 min. resazurin test passes nearly 50% of unsatisfactory milks.

5. The data recorded in this paper all go to show that the best indirect index of the K.Q. of raw milk is afforded by the methylene-blue test as carried out by the method officially laid down by the Ministry of Health.

6. On the other hand, if time is an overruling consideration, then a slightly less accurate estimate of the K.Q. of raw milk is afforded by the 1 hr. resazurin test. It must be pointed out, however, that the figures given in Table 6 suggest that below disk 2 there is some irregularity in the relation between the disk numbers and the K.Q. If the 1 hr. resazurin test should come into use for the routine grading of milk, a revision of the present tints at the lower end of the scale might prove desirable.

We would express our thanks for technical assistance in this investigation to Miss Burt, Miss Pearman, and Miss D. M. Elliott; and to the Directors of United Dairies, Ltd., for permission to publish the results.

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318. THE EFFECT OF ACIDITY ON THE PRODUCTION OF DIACETYL BY BETACOCCI IN MILK

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(With 4 Figures)

Cultures used as butter starters contain both acid-forming streptococci and citric-acid-fermenting organisms (betacocci), and are generally known in New Zealand as mixed starters. In these mixed starters the betacocci (*Str. citrovorus* and *Str. paracitrovorus*) are mainly responsible for diacetyl production at 21° C. Whereas most pure cultures of the betacocci do not produce diacetyl in milk at its normal *p*H value, in mixed starters diacetyl production by the betacocci is favoured by the acid produced by the lactic streptococci. Wiley, Cox & Whitehead (1) showed that the diacetyl content of a mixed starter is constantly altering, the maximum production at 21° C. being reached in approximately 12 hr. after inoculation, followed by rapid destruction of most of the diacetyl. Michaelian, Hoecker & Hammer (2) investigated the effect of various acids on the production of diacetyl plus acetoin by single strains of betacocci growing at different *p*H values, and showed that highest yields were obtained with citric acid as an acidulant. Van Beynum & Pette (3) showed that the betacocci produced acetic acid and carbonic acid in non-acidified milk, but that in acidified milk diacetyl, acetoin and 2–3-butylene glycol were formed in addition. The proportion of the various compounds formed depended on the acidity. It is thus evident that the acidity of the medium in which the betacocci are grown is a factor of major importance in relation to their diacetyl production. The present paper describes the results obtained when single strains of betacocci were grown in milk adjusted to various *p*H values.

Sources of the betacocci and their biochemical reactions

During the course of the investigation eleven strains of betacocci were examined (Table 1). Seven of these (strains Beta 1–Beta 7) were obtained by the method of Hammer (4) from mixed starters prepared from commercial powder cultures, in some cases two strains (showing slight morphological differences) being obtained from the same mixed starter. Two of the strains of betacocci (12*b* and RB) had been in stock in the laboratory for several years, having been obtained initially from mixed starters. The two other strains (Beta S1 and T13) had been isolated from rye-grass and cheese respectively (5). Some of the biochemical reactions of the betacocci are shown in Table 1. They all produced diacetyl, and all showed increased acid production with yeast extract. The addition of citric acid invariably resulted in a distinct increase in the volatile acid produced. Most of the strains showed only a slight colour change when grown in litmus milk for 7 days at 21° C.

Methods of analysis and procedure

Volatile acidities were determined by the method of Hammer & Bailey (6), the results being expressed as the number of ml. of *N*/10 NaOH required to neutralize 1 l. of distillate from 250 g. of culture. Diacetyl was estimated by the method of Pien, Baisse &

Martin(7) with a modification introduced by Cox & Wiley(8). The *p*H values were determined electrometrically using a quinhydrone electrode. Acidities were estimated by titration with *N*/10 NaOH, using phenolphthalein as indicator.

For each experiment skim milk was flash pasteurized in a conical flask by heating to 85°C. and then cooling immediately. It was then distributed in 500 ml. quantities to four flasks. These were adjusted to different acidities by the addition of sterile diluted lactic acid, the total volumes being kept the same by the addition of appropriate quantities of water. All the flasks were inoculated with 5% of a 24 hr. culture of a betacoccus which had been grown at 30°C. After aeration(9), 75 ml. quantities were distributed to small conical flasks which were then incubated at 21°C. From time to time flasks were removed from the incubator and analyses for diacetyl were carried out. Acidities and *p*H values were determined at intervals.

Table 1. *Biochemical reactions of the betacocci*

Strain	Di-acetyl pro-duction	Effect when grown in litmus milk for 1 week at 21°C.	Rise in acidities as % lactic acid after 1 week at 21°C.		Volatile acid. ml. *N*/10 NaOH to neutralize 1000 ml. distillate from 250 g. culture		Source of strains
			Milk	Milk + yeast extract	Milk	Milk + citric acid	
Beta 1	+	Slight colour change	0·05	0·09	10·4	49·6	Mixed starter from commercial powder culture A
2	+	Slight colour change	0·03	0·60	6·8	25·4	Mixed starter from commercial powder culture B
3	+	No colour change	0·01	0·06	—	56·3	Mixed starter from commercial powder culture C
4	+	Slight colour change	0·05	0·61	—	58·9	
5	+	Slight colour change	0·07	0·61	15·0	38·5	Mixed starter from commercial powder culture D
6	+	Slight colour change	0·03	0·34	11·1	39·2	
7	+	Slight colour change	0·03	0·31	8·3	30·7	Mixed starter from commercial powder culture E
12*b*	+	Slight colour change	0·05	0·77	12·8	24·2	Mixed starter
RB	+	No colour change	0·01	0·12	16·9	52·9	Mixed starter
Beta S1	+	Pink	0·08	0·63	14·2	63·3	Perennial rye-grass
T13	+	Pink	0·19	0·66	23·4	48·5	Cheese

Diacetyl production by the betacocci

In each experiment the betacocci were grown at different *p*H values represented by the letters A, B, C and D. The approximate acidities and *p*H values are shown in Table 2.

Table 2

Flask	Acidity % lactic acid	*p*H value
A	0·43	5·5
B	0·56	5·0
C	0·63	4·7
D	0·72	4·4

Results showed that the betacocci produced diacetyl and then partly destroyed it, the rate of production and destruction of diacetyl depending largely on the *p*H value. In Fig. 1 are shown graphs drawn for strains Beta 2 and Beta 3. These graphs are typical in a general way of the results found for strains Beta 1–Beta 7. Amounts of diacetyl up to a maximum of about 20 p.p.m. were formed. Strain 12*b* (Fig. 1) produced very little diacetyl, less than 3 p.p.m. being formed. Strain RB (Fig. 2) produced and destroyed

diacetyl quickly at the *p*H values shown, 23 p.p.m. of diacetyl being produced at *p*H 4·4 in a relatively short time. In one experiment this strain produced 40 p.p.m. of diacetyl and subsequently partly destroyed it. Strain Beta S1 (Fig. 3) possessed the greatest diacetyl-producing power of the strains examined, 60 p.p.m. of diacetyl being produced and subsequently destroyed at *p*H 4·4. Strain T13 produced 20 p.p.m. of diacetyl at

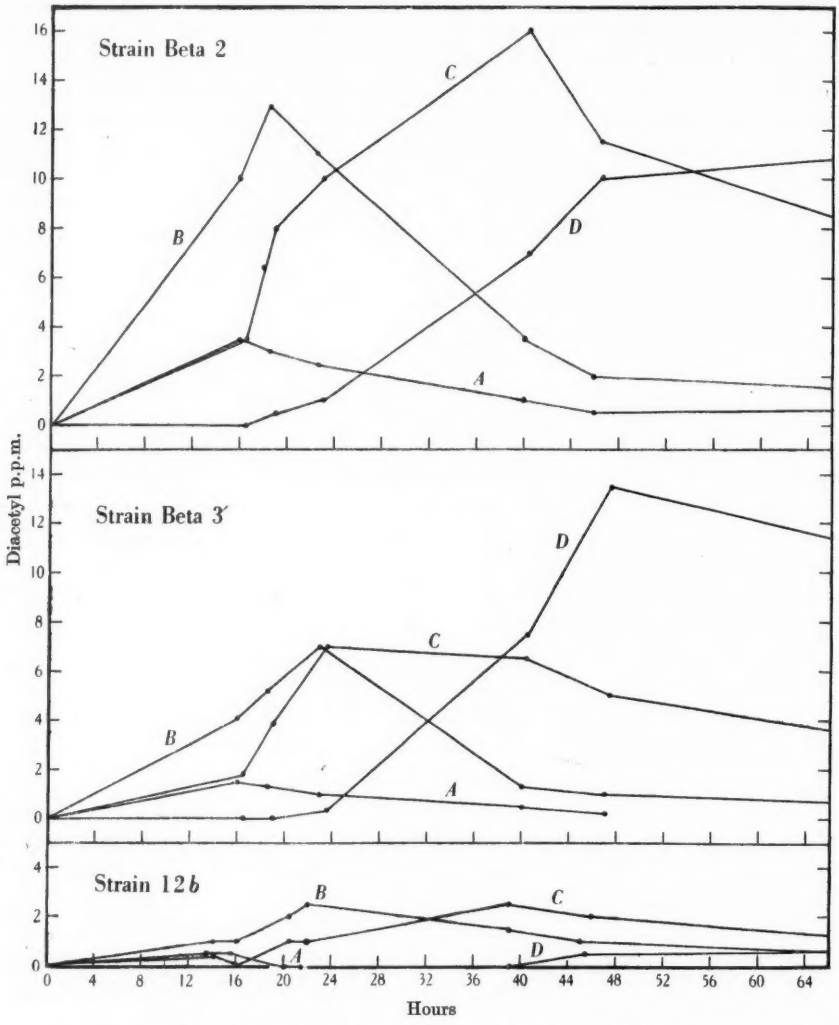

Fig. 1. Diacetyl production by betacocci at various *p*H values.

*p*H 4·4 on prolonged incubation. At the other *p*H values only small amounts of diacetyl were produced.

It must be borne in mind that, as analyses were done only at intervals, a complete picture of diacetyl production and destruction could not be obtained, and certain peaks on the graphs may have been missed. Nevertheless, the results obtained were sufficient

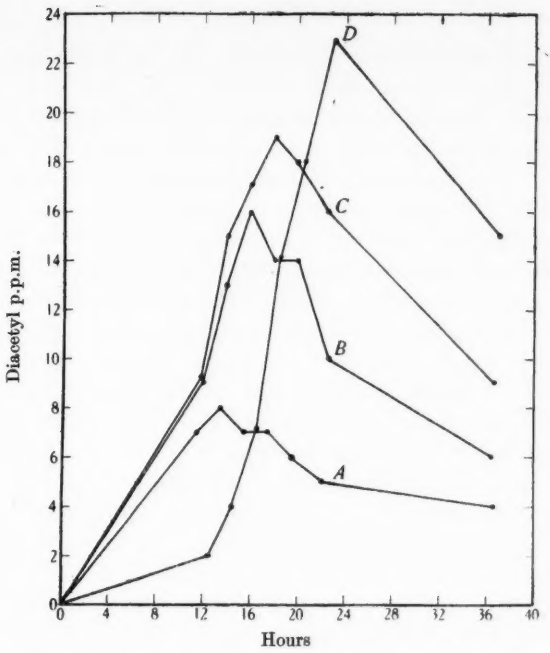

Fig. 2. Diacetyl production by strain RB at various *p*H values.

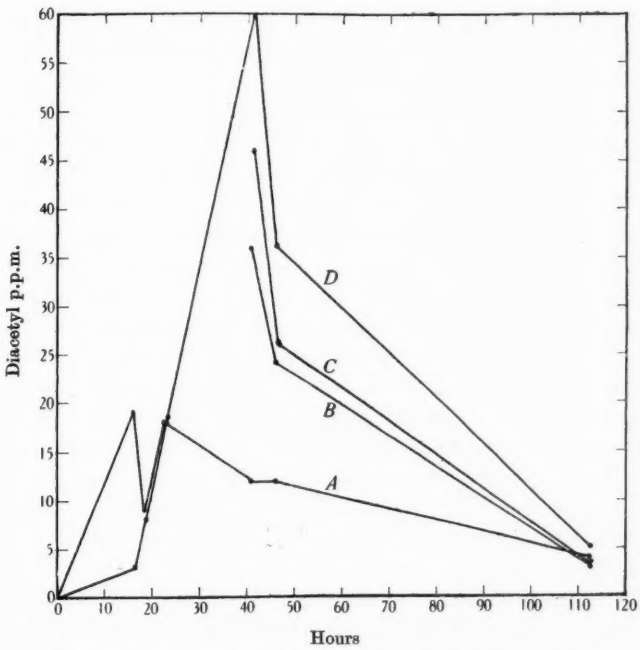

Fig. 3. Diacetyl production by strain Beta S 1 at various *p*H values.
(Drawn to smaller scale than Figs. 1 and 2.)

to indicate in a general way the diacetyl-producing powers of the strains of betacocci examined.

An attempt had been made to standardize the quantity of inoculum by using a 24 hr. culture which had been inoculated with a 48 hr. culture grown at 30°C. This method did not overcome the difficulty due to some organisms growing faster than others, thereby resulting in a variation in the total numbers of organisms added in different experiments. Wiley *et al.*(1) showed that with inoculations of 1·0 and 0·001% of a mixed starter the same types of graphs were obtained, the use of the smaller quantity of inoculum delaying the maximum production of diacetyl by about 15 hr. Although conditions for the growth of betacocci at fixed *p*H values are different from the conditions obtaining in a mixed starter, these findings indicate that there could be fairly wide variations in the numbers of betacocci used for inoculation without greatly altering the results obtained. Hence it was considered that the graphs described would be sufficiently characteristic of the diacetyl-producing powers of the strains examined.

In general it was observed that the lower the *p*H values at which the betacocci were grown, the greater was the lag period before considerable diacetyl was formed. The lowered *p*H had a restraining effect on the rate of growth of the organisms. This was confirmed by frequent microscopic examinations. Owing to the curdled condition of the milk at the lower *p*H values it was not possible to do accurate counts of the numbers of organisms, and a rough method had to be adopted. A loopful of the milk to be examined was spread on a slide, dried, and stained, and the growth as observed microscopically was recorded as good, poor, or otherwise. An example of the results is shown in Table 3.

Table 3. *Growth of betacocci as observed microscopically for strain Beta* 3

Flask	Lactic acid %	*p*H value	Time after inoculation				
			16 hr.	19 hr.	23 hr.	40 hr.	112 hr.
A	0·43	5·5	Good	Good	Good	Good	Good
B	0·58	5·0	Fair	Fair	Good	Fair	Fair
C	0·64	4·7	Poor	Poor	Poor	Fair	Fair
D	0·74	4·4	Very poor	Very poor	Very poor	Poor	Poor-Fair

The results showed that the metabolism of the organisms was altered by the lowered *p*H. At the lower *p*H values growth took place with difficulty, and some strains showed a tendency to produce involution forms. The rate of growth of the organisms was progressively slower the lower the *p*H of the milk. Yet the amount of diacetyl formed at the lower *p*H values was as great as that formed at the higher *p*H values and sometimes greater, showing that the power of production of diacetyl per unit cell increased as the *p*H fell, which pointed to a definite change in the metabolism of the organisms. Van Beynum & Pette(3) pointed out that the higher the acidity the greater was the amount of diacetyl and the smaller was the amount of acetic acid formed. Table 3, taken in conjunction with the graph for strain Beta 3, indicates a progressive alteration in the metabolism of the organisms with decreasing *p*H, and is confirmatory of the work of Van Beynum & Pette.

All strains were not affected to the same extent by *p*H changes in the medium. Decrease in *p*H down to 4·4 affected the growth of strain RB less than that of other strains, but at the same time the metabolism of this organism was affected somewhat, Fig. 2 showing that the diacetyl production increased as the *p*H was lowered. Strain RB

showed a quicker production and destruction of diacetyl than most other strains, due no doubt to its being less retarded in growth by decrease in *p*H. It grew vigorously under ordinary conditions and this would help it to withstand certain decreases in *p*H better than organisms which grew only poorly. Strain Beta S1 also grew vigorously under ordinary conditions and showed a fairly quick production and destruction of diacetyl at all the *p*H values investigated. Decreases in *p*H affected the rate of growth of this organism more than that of strain RB. Although analyses were not carried out at maximum concentrations (Fig. 3), it was evident that the metabolism of strain Beta S1 changed with decreased *p*H.

Discussion

An attempt was made to correlate the diacetyl-producing powers of the betacocci with other chemical reactions. No relationship could be established, however, between diacetyl production by the organisms and their acid production in milk, their acid production in milk plus yeast extract, and their volatile acid production. Nor could any relationship be found between diacetyl production and sugar fermentations when the organisms were classified according to the method of Hucker & Pederson (10). The diacetyl-producing powers of the betacocci appeared to be characteristics unrelated to the other biochemical properties of the organisms.

The changes in metabolism which the betacocci undergo at different *p*H values throw light on the diacetyl changes in mixed starters. The diacetyl content is constantly altering both in mixed starters and in cultures of single strains of betacocci growing at various fixed *p*H values. Hence it is necessary to do analyses from time to time in order to follow the diacetyl production and destruction by single strains of betacocci. Michaelian *et al.* (2) worked at *p*H values ranging from 5·3 to 2·5 and found maximum diacetyl production at *p*H values of about 4·0, but, as their analyses were done at only one time, viz. 48 hr. after adjustment of *p*H values, diacetyl quantities may have been altering at that time, and a true picture of preceding events could not be obtained. In mixed starters maximum amounts of diacetyl were produced in about 12 hr., a *p*H of less than 4·4 being reached (1), but some typical strains of betacocci grown at *p*H 4·4 took more than 40 hr. to attain maximum diacetyl production, there being practically no production of diacetyl during the first 20 hr.

It would appear that a steady decrease in *p*H produced conditions more favourable for rapid diacetyl production by the betacocci than a *p*H kept at a constant level.

An experiment was carried out to find the effect on diacetyl production of altering the *p*H from time to time. The results are shown in Fig. 4. Milk was flash pasteurized, cooled, acidified to about *p*H 5·5 (*M*), inoculated with 0·2% of a 24 hr. culture of strain Beta 3, aerated, and distributed to flasks in 100 ml. quantities. These were incubated at 21°C. and analysed at intervals. When an analysis was carried out several flasks were reserved for further diacetyl estimations at that particular *p*H, and the remaining flasks were acidified to a lower *p*H. In this way the course of diacetyl production could be followed with changing *p*H values (curve *M'N'O'P'*). At the same time the diacetyl contents at the fixed *p*H values *M* (5·5), *N* (5·1), *O* (4·8), and *P* (4·5) were followed.

The curve *M'N'O'P'* shows a fairly rapid increase in the amount of diacetyl, and the experiment thus proves that the constant addition of acid to the organisms caused the diacetyl content to increase rapidly. Previous experiments have shown that under

similar conditions the high diacetyl content at *p*H 4·4 would not have been reached till 30–40 hr. after the commencement of the experiment.

These results enable an explanation of the rapid production of diacetyl in mixed starters to be offered. When cultures of mixed starters are inoculated into milk and incubated at 21°C. the betacocci and the streptococci multiply for some time before there is marked alteration in the *p*H of the medium. Then the acid produced by the lactic streptococci begins to influence the betacocci; their growth is retarded, but their

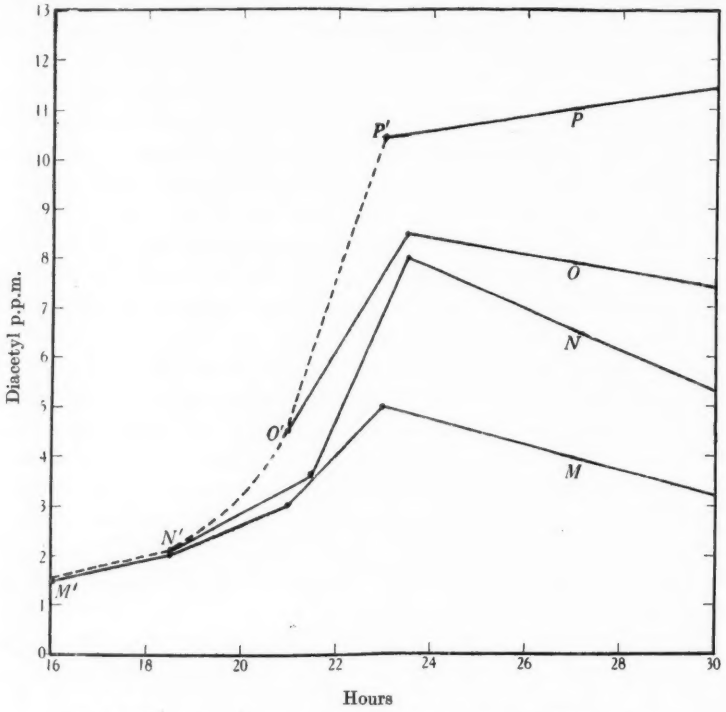

Fig. 4. The effect of repeated additions of acid on diacetyl production by strain Beta 3.

power of diacetyl production per unit cell is increased. Hence there will be a rapid rise in the diacetyl content of the starter because the large number of betacocci which had been produced initially now have increased power of diacetyl production per unit cell. There are thus in the mixed starter more diacetyl-producing cells as the *p*H reaches low values than there are in a pure culture of betacocci kept at a low *p*H from the start. On the other hand, a pure culture of betacocci kept at a high *p*H would show more active cells, but those cells would not produce much diacetyl; hence the different diacetyl curves shown by mixed starters and by pure cultures of betacocci at various *p*H values.

Summary

1. The diacetyl production of betacocci isolated from starters, cheese and rye-grass was investigated at *p*H values ranging from 5·5 to 4·4. The betacocci produced diacetyl and subsequently partially or wholly destroyed it.

2. There was a considerable variation in the amount of diacetyl produced by the different strains of betacocci. Capacity to produce diacetyl could not be correlated with any other property of the organisms.

3. The rate of growth of the organisms was progressively slower the lower the *p*H, but at least as much diacetyl was eventually formed at low *p*H values as at high *p*H values. The results showed that there was an alteration in the metabolism of the organisms, the diacetyl-producing power per unit cell increasing with decreasing *p*H.

4. The results found enable an explanation of the rapid production of diacetyl in mixed starters to be offered.

The author is indebted to Dr H. R. Whitehead for helpful advice and criticism.

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319. THE FORMATION OF CARBON DIOXIDE BY LACTIC ACID BACTERIA AND *BACILLUS LICHENIFORMIS* AND A CULTURAL METHOD OF DETECTING THE PROCESS

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Among the bacteria certain facultative anaerobes, when fermenting carbohydrates and other substances, form large amounts of carbon dioxide but no other gaseous product. This feature of the organisms usually escapes detection in the visual examination of cultures on the ordinary bacteriological media; the CO_2, owing to its considerable solubility in water and its passage into the atmosphere, commonly fails to saturate the medium. Consequently visible gas formation in agar or in Durham, Einhorn or Smith fermentation tubes is generally inconspicuous and, depending on several factors, erratic in occurrence.

The production of CO_2 during the anaerobic breakdown of sugars has probably received more attention as a diagnostic character among the lactic acid organisms than in other groups of the bacteria. The heterofermentative streptococci and lactobacilli may be

distinguished from homofermentative species by the larger amounts of gas which they produce. For detecting the gas-forming ability of these organisms a quantitative estimation of CO_2 seems to be universally accepted as the only reliable procedure. The chemical method is laborious for diagnostic purposes, and many attempts have been made to find a quick test which makes use of the evolution of visible gas. Leichmann (1) initiated the separation of gas in whey gelatin cultures by thrusting an inoculating wire into the medium, apparently utilizing the effect of forming nuclei of gas in a supersaturated solution. Burri (2) used the same method with agar-shake cultures, and Hammer & Baker (3) plunged a hot wire into milk cultures. In order to demonstrate and measure the gas evolved from cultures Freudenreich (4) employed a eudiometer tube and Allen (5) a capillary tube containing a mercury pellet. Orla-Jensen (6) used heavily seeded agar-shake cultures, and Hammer & Baker (3) experimented with wax and vaseline seals for milk cultures. Struyk (7) designed a fermentation tube capable of retaining CO_2. Several of these methods require special apparatus, and of the others none appears to be reliable for demonstrating gas production by all strains of heterofermentative lactic acid bacteria.

While studying *Bacillus licheniformis* (Weigmann) Gibson (8), an organism which does not normally show gas formation in media of the usual composition, we found that increasing the concentrations of glucose and peptone to 5% enabled the bacillus to produce considerable amounts of visible gas. The gas production was most active if the medium was distinctly alkaline (*p*H 8–9) at the time of inoculation. Still larger quantities of gas were evolved in milk + 5% glucose. The gas formation could be demonstrated in deep agar-shake cultures, or in liquid media covered with a layer of agar or a wax-petroleum jelly mixture, or in V-shaped fermentation tubes. These observations add to the criteria available for differentiating *B. licheniformis* from *B. subtilis*. The two species resemble each other in general character and have frequently been confused (8), but *B. subtilis* fails to produce visible gas except, in the case of certain strains, when provided with nitrate. It seemed likely that a similar simple test might be evolved for differentiating the heterofermentative from other types of the lactic acid bacteria and this has been realized.

The production of gas from sugars by the heterofermentative lactic acid bacteria

This investigation has shown that several factors influence the formation of CO_2 and that they must all be controlled before consistent results with every strain of the organisms can be anticipated in a test depending upon visible gas production. Neglect to provide one or more of the factors appears to be the explanation of a belief that lactic acid bacteria behave erratically as gas producers.

Some of the exploratory work was done using a simple test-tube technique described later in this paper. Much use was also made of straight-sided, V-shaped fermentation tubes about 1·2 cm. in diameter with a 10 cm. closed limb and a 20 cm. open limb at an angle of about 30° to each other. This tube facilitates work in which constituents of the media must be mixed after sterilization, for the medium is easily introduced aseptically so as to fill the closed arm. Gases are also well retained, especially if the exposed surface of the medium is covered with a layer of sterile agar.

The following factors were found to promote the formation of CO_2 from sugars by the heterofermentative lactic acid organisms:

(1) *The use of a suitable sugar.* All strains form gas readily from glucose. A proportion of those which ferment sucrose were tested with that sugar and the results appeared to be identical with those obtained with glucose. Lactose, on the contrary, never yielded CO_2 rapidly or in large amounts, and various lactose-fermenting strains failed to produce visible gas from that sugar under any of the conditions tried. Since the work of Orla-Jensen(6) it has been recognized as a feature of the lactic acid bacteria that they do not form acid with equal facility from different sugars, and it is clear that the amount of CO_2 produced also varies with the substrate. The peculiar unsuitability of lactose as a source of CO_2 may be partly responsible for the frequently described variability of the gas production by these organisms in milk and its derivatives.

(2) *The reaction and the buffering capacity of the medium.* It might have been anticipated that more CO_2 would be liberated from a culture which becomes strongly acid than from one which remains closer to neutrality, but this does not apply to the organisms considered here. Tests were made with broths having peptone concentrations up to 5%, nutrient gelatin (with 12% gelatin) and skim milk as the basal ingredients of the media. In some trials 0·5% K_2HPO_4 was also added. Little visible gas was formed even by the most active strains in poorly buffered but otherwise favourable media, while the greater the buffering capacity of the medium the larger was the amount of CO_2 evolved. As the basis of a medium for detecting gas formation the strongly buffered materials, milk and nutrient gelatin, are both reliable and approximately equal in suitability. Their buffer curves were found to run close to each other over the *p*H range of the cultures. Still larger volumes of gas are liberated from a medium containing 20% gelatin, the highest concentration of this substance which was tested. The value of these media in promoting the liberation of visible gas is probably not entirely attributable to their influence on the reaction. Their capacity to absorb measured volumes of pure CO_2 was tested roughly in V-tubes. After passing the gas into the closed limb the tubes were agitated uniformly at 30° C. (a temperature at which the gelatin preparations are liquid), and it was noted that milk and gelatin, and especially 20% gelatin, absorbed the CO_2 less rapidly and to a smaller extent than more dilute solutions.

The optimum initial reaction is close to neutrality; the more acid the medium at the time of inoculation the smaller is the volume of gas formed, while on the alkaline side of *p*H 7 growth does not start readily.

Both factors, the original neutrality and the buffering capacity of the medium, prolong the period of cell proliferation (as measured by plate counts), but it is possible that they also influence the metabolism of the organisms. Yeasts, in which the fermentation is known to be modified by the reaction, show little gas formation in neutral or alkaline cultures, the effect of reaction on the amount of CO_2 formed thus being different from that noted in the case of the lactic acid bacteria.

It may be recalled that Sherman(9) observed that strongly buffered media promote the formation of CO_2 by *Streptococcus kefir*, and the influence of gelatin has evidently been noticed before; Orla-Jensen(6) and Hucker & Pederson(10) give illustrations of betacocci in sucrose gelatin some of which show gas formation.

(3) *Activating factors.* The inclusion in the medium of yeast infusion, yeast autolysate or tomato juice has a markedly stimulative effect on the formation of CO_2 from sugars

by the heterofermentative lactic acid bacteria. If one of these preparations or some alternative material is not added to glucose milk or glucose gelatin, visible gas either fails to appear or does so in small amounts only. Active gas formation by streptococci can be ensured by adding yeast infusion or 0·25% of the commercial yeast autolysate yeastrel, but other substances may be necessary for certain lactobacilli. The requirements of the weakest gas formers in the latter group appear to be satisfied by a supplement of 0·25% yeastrel + 10% tomato juice (the juice of canned tomatoes squeezed through muslin, filtered through paper pulp and adjusted to *p*H 6·5, or a similar juice prepared from the fresh fruit). This combination is also slightly more favourable to gas formation by streptococci than is yeast infusion, yeast autolysate or tomato juice alone. The concentrations just mentioned are about the lowest which will produce a maximal effect; double the amounts give similar results. The efficacy of tomato juice becomes reduced on storage; a specimen which had been kept for a year in a screw-capped bottle had to be used in a concentration of 40% in order to give the same effect as 10% of fresh juice.

On account of a war-time scarcity of tomatoes, cabbage juice was tried and found to be equally effective. To obtain the juice the roughly chopped leaves are pressed into the empty inner vessel of a double pot while the outer vessel contains boiling water; after heating for 15 min., when the tissue has softened, the juice is expressed, filtered and adjusted to *p*H 6·5.

These materials are known to be rich sources of growth factors required by lactic acid bacteria, but it seemed uncertain that their great influence on the formation of CO_2 was due simply to the promotion of extra growth. In order to elucidate this point a strain of *Streptococcus kefir* was cultivated in nutrient gelatin containing 1 and 7·5% glucose, in each case with and without the addition of 0·5% yeastrel, and plate counts were made at intervals during a period of 5 days. The yeastrel produced a three- to fourfold increase in the volumes of gas liberated, but it nevertheless retarded the multiplication of the organism, reduced the maximum cell population attained, and accelerated death of the cells. Evidently the effect of yeast preparations, in the optimal concentrations for gas formation, is mainly at least a specific one on the rate of some phase of the fermentation and not due to enhanced growth. Further evidence supporting this conclusion was obtained when the conditions influencing the fermentation of citrate were investigated. Yeast extracts were found to have only a small effect on the formation of CO_2 from citrate by organisms which show a strong response when fermenting glucose. Analogous observations have been made by Braz & Allen (11). They found that a yeast autolysate (marmite) promoted acid formation in milk, but did not increase the maximum numbers of organisms which developed and tended to decrease their viability. In contrast to their influence on the lactic acid bacteria the factors in yeast and plant juices have little effect on CO_2 production by *Bacillus licheniformis* in glucose milk or glucose nutrient gelatin.

(4) *The concentration of sugar.* To permit the maximum production of gas the sugar requirement of the organism must be satisfied. It has, however, been found that the accumulation of CO_2 is promoted by still greater amounts of sugar. The optimum for glucose was first reached with concentrations varying from 3 to 7·5% depending on the organism, and inhibition was not evident until 7·5% was exceeded. Estimations of residual sugar in glucose yeast gelatin cultures of a strain of *Streptococcus kefir* incubated for 4 days, the period during which the most active formation of gas occurs, showed

that the organism had utilized the glucose in concentrations up to about 2% only. So far the only effect promoting the evolution of gas which has been recognized as attributable to higher concentrations of glucose is that of decreasing the solubility of CO_2 in the medium, but that this is the only factor concerned seems doubtful.

A CULTURAL METHOD OF DETECTING THE FORMATION OF CARBON DIOXIDE FROM GLUCOSE

The foregoing discussion shows that CO_2 is formed actively by the heterofermentative lactic acid bacteria in glucose (5%) milk or glucose (5%) nutrient gelatin (containing 10–12% gelatin), each supplemented with a preparation from yeast and the juice of tomato or cabbage. Either medium may be used to differentiate the heterofermentative lactic acid bacteria and *Bacillus licheniformis* from organisms with which they might be confused. For streptococci the plant juice is not essential, and for the spore former the yeast extract may also be omitted. If nutrient gelatin is prepared solely for the purpose of this test, it may with advantage be made with 20% gelatin, filtration being omitted.

In order to detect the gas visually it is only necessary to ensure that it shall be adequately retained within the culture. The V-tube already described may be used, but the following simple technique, employing the ordinary straight tube available in any laboratory, is equally reliable:

If milk is to be used, the glucose, yeast extract and plant juice are added to a mixture of four parts separated milk and one part nutrient agar, and the medium is distributed in tubes and sterilized by intermittent steaming. When gelatin is employed in place of milk there appears to be no advantage in adding agar, even if a temperature above the melting-point of gelatin is to be used for incubation.

To make a test the medium is liquefied, cooled to 45° C., inoculated and chilled; after the gel has formed, melted and cooled nutrient agar is gently poured into the tube so that it forms a layer 2–3 cm. deep above the gelatin or soft milk agar. The culture is then incubated at approximately the optimum temperature for the organism and observed for gas formation.

Where the method is used only occasionally it is convenient to make the necessary additions to tubes of litmus milk or gelatin by pipette. A 50% solution of glucose and the other special ingredients can be separately sterilized in the autoclave. Screw-capped bottles are suitable containers as they eliminate evaporation. Prepared in this way the medium is less acid than when sterilized complete, and it is slightly more favourable to gas formation, but the difference is unimportant. On no account should the completed medium be sterilized in the autoclave.

The amount of gas formed is proportional to the depth of the medium, and it would therefore be inadvisable to use shallow layers. In this work the tubes have been filled to a depth of 5–6 cm. exclusive of the agar seal.

An essential condition of the test is rapid growth of the organism, presumably in order to avoid a gradual dissipation of the gas. Consequently the temperature of incubation should not be greatly different from the optimum for the species. A relatively large inoculum results in earlier and more vigorous gas production than does a light seeding, and in the case of organisms which grow feebly on artificial media (e.g. certain lactobacilli) it is an advantage to inoculate heavily by pipette from a young active culture.

Plate counts indicate that neither the glucose, gelatin nor yeast extract, in the concentrations suggested, have singly a beneficial influence on cell proliferation as distinct from gas formation, but when the ingredients are combined the complete medium is not unfavourable to growth. No difficulty has been experienced owing to a failure of the organisms to initiate growth.

This technique of detecting CO_2 appears to have three main features: (*a*) the use of a medium which promotes gas formation, (*b*) the relative insolubility of CO_2 in the medium, and (*c*) the adequacy of gas retention. The combination of gelatin or soft agar with a solid agar closure entraps CO_2 more efficiently than shake cultures in either semi-solid or normal strength agar alone. The gelatin or soft agar probably act by hindering convection currents; and when gas is evolved the solid agar tends to separate intact from the underlying medium and to be forced up the tube like a piston.

The reliability of the cultural test: the organisms examined

Special attention has been given to the lactic acid bacteria, as it is in this group that the test for CO_2 production is likely to have its main application. Representatives of a number of the better-known species of these organisms have been subjected to the test. Some are recognized type cultures; the remaining strains appear to have been reliably identified or characterized either by ourselves or by others. The majority are streptococci which were isolated during a study of the microflora of milk, an investigation in which the test for gas formation proved to be a useful diagnostic tool.

The following heterofermentative organisms have consistently formed gas in the test: 334 strains of *Streptococcus kefir*, 15 of *S. citrovorus*, 5 of *Leuconostoc mesenteroides*, 2 of *Lactobacillus brevis* and 6 of an unidentified type of *Lactobacillus* from silage. The six unidentified lactobacilli were isolated and studied by Cunningham & Smith and are strains of their group V which is characterized (12) by a feeble growth on artificial media and a low yield of fermentation products. Cunningham & Smith found that although the organisms give low ratios of lactic to acetic acid the quantities of CO_2 produced in a glucose yeast extract tryptone broth are small and in the case of certain strains are comparable with the amounts yielded by homofermentative species. It is therefore believed that these organisms provide a severe test of the qualities of the media and the reliability of the cultural test described in this paper. They do not liberate visible gas rapidly and the volumes produced are comparatively small, but a negative result has not been experienced. With the exception of certain strains of *Streptococcus citrovorus* all the other heterofermentative organisms which have been examined are more vigorous gas producers.

Slight gas formation in milk cultures of homofermentative lactic acid bacteria has sometimes been described, and it is not uncommon to detect streaks on a curd which suggest that gas bubbles have been produced. Nevertheless, definite bubbles have never been detected when the test described in this paper has been applied. The homofermentative organisms which have been tested are: 12 strains of streptococci of Lancefield's group A, 1 of group C and 1 of group G, 48 of *Streptococcus agalactiae*, 3 of *S. dysgalactiae*, 1 of *S. uberis*, 7 of *S. salivarius*, 55 of *S. bovis*, 77 of *S. thermophilus*, 49 of *S. lactis*, 13 of *S. cremoris*, 52 of *S. faecalis*, 13 of *S. liquefaciens*, 3 of *S. zymogenes*, 2 of *S. durans*, 232 of a non-haemolytic variant of *S. durans*, 41 of an unidentified thermoduric strepto-

coccus of Lancefield's group D, 1 of an unidentified streptococcus of group D with a comparatively low resistance to heat, 1 of *Lactobacillus bulgaricus*, 1 of *L. acidophilus*, 1 of *L. casei*, 2 of *L. plantarum* and 2 of *L. delbrueckii*.

Thus, a total of 362 strains of heterofermentative types produced visible gas in the cultural test while 618 strains of homofermentative species failed to do so, and it may be inferred that as a means of differentiating the two groups the method possesses adequate precision.

A recent paper by Smiley, Niven & Sherman (13) describes how the growth of *Streptococcus salivarius* in a synthetic glucose-containing medium is not readily initiated unless the glucose is sterilized in the medium; the addition of pyruvic acid results in prompt growth when glucose is sterilized separately, and if sufficient pyruvic acid is added visible gas may be formed. In view of these findings and the possible effects of sterilization on media containing high concentrations of glucose, it seemed desirable to examine further the gas-forming ability of homofermentative species. Using the media and methods described above it was found that the addition of pyruvic acid to the glucose milk or glucose gelatin enabled certain strains of *S. salivarius*, *S. agalactiae*, *S. bovis*, *S. faecalis*, *S. liquefaciens*, *S. zymogenes* and *S. durans* to produce small amounts of visible gas. Pyruvic acid was, however, ineffective in concentrations lower than about 0·5%. Tests were also made with *S. salivarius* in media which had received an excessive heating. The organism was grown in 5% glucose milk and 5% glucose gelatin which had been autoclaved at 15 lb. for 30 min. and neutralized before inoculation, but the cultures showed no formation of visible gas. These results indicate that pyruvate or other products of the breakdown of sugars are not formed during sterilization in amounts sufficient to affect the differential value of the test for gas production.

THE FORMATION OF CARBON DIOXIDE FROM CITRATE BY LACTIC ACID BACTERIA

The agar-closure method of detecting gas formation may be employed to demonstrate the fermentation of citrate by lactic acid bacteria. Certain homofermentative species can produce visible gas from citric acid in media containing no carbohydrate, while heterofermentative species utilize citric acid only in the presence of a fermentable sugar. The findings described below indicate that gas production from citrate has some value as a differential criterion, and they are in general agreement with those already described by Campbell & Gunsalus (14) concerning the species which utilize citrate as a source of energy.

The reaction of the medium is an important factor controlling the formation of CO_2 from citrate. In a citrate medium with a *p*H value of 7 or higher the citric acid fermenters either fail to initiate growth or, if growth does occur, as in the case of enterococci, little or no visible gas is liberated. The optimum initial reaction of the medium appears to be in the range of *p*H 5·5–6·0 for at least most of the citrate fermenters. In this work a mixture of tribasic potassium citrate and citric acid in the proportions of 9 parts to 1, which possesses considerable buffering capacity around *p*H 6, has been found a convenient source of citrate. The mixture is added to media before sterilization, or as an autoclaved 50% solution later, to give a concentration of approximately 3%. A concentration of 5% appears to be equally suitable for enterococci, but may be inhibitive to other species.

Homofermentative species. A suitable general medium for the homofermentative group is nutrient gelatin + 3% citrate-citric acid mixture + 0·25% commercial yeast extract + 10% tomato or cabbage juice. Various broths may be superior for certain organisms, but are not so for others. Milk, as the basis of a medium, is unfavourable to citrate utilization in the case of organisms which produce acid rapidly. If homofermentative streptococci only are being examined, the yeast and plant addenda may be omitted from the citrate gelatin without altering the results.

Streptococci of the enterococcus group which have been found to produce gas in citrate gelatin are: 51 strains of *Streptococcus faecalis*, 13 of *S. liquefaciens*, 3 of *S. zymogenes*, 2 of *S. durans*, and 100 of a non-haemolytic variety of *S. durans*.

Homofermentative streptococci which have failed to show gas formation from citrate are: 11 strains of Lancefield's group A, 1 of group C and 1 of group G, 16 of *S. agalactiae*, 3 of *S. dysgalactiae*, 1 of *S. uberis*, 7 of *S. salivarius*, 18 of *S. bovis*, 23 of *S. thermophilus*, 26 of *S. lactis*, 6 of *S. cremoris*, 10 of an unidentified thermoduric streptococcus of Lancefield's group D, and an unidentified streptococcus of group D with a comparatively low heat resistance. The two latter types, the systematic status of which is problematical, possess affinities with the enterococci as will be described in a subsequent publication, but inactivity towards citrate and other distinctive features appear to justify their separation from the well-characterized members of that group. Gas formation from citrate thus promises to be a useful diagnostic character of the enterococci, although the production of acetylmethylcarbinol from citrate, which will be described in a future paper, serves the same purpose and may be found to be more convenient to detect.

Homofermentative lactobacilli which have been found to liberate visible gas are a strain of *Lactobacillus casei*, two strains received as *L. casei* ϵ and *L. helveticus* and two unidentified strains which do not ferment lactose (*L. leichmannii*?). Negative results were given by one strain of *L. bulgaricus*, one of *L. acidophilus*, two of *L. plantarum* and two of *L. delbrueckii*. An examination of a more extensive series of strains is obviously necessary, particularly with regard to the distinction between *L. casei* and *L. plantarum* which the test may provide.

Heterofermentative species. The formation of gas from citric acid by heterofermentative species may be demonstrated in the citrate gelatin medium already described if it is supplemented with 0·5% glucose. Lower concentrations of sugar do not ensure the liberation of visible gas by every strain. As CO_2 is formed from both sugar and citrate, the test should be controlled by a parallel culture without citrate.

All the heterofermentative organisms which have been tested have produced visible gas from citric acid. They comprise 99 strains of *Streptococcus kefir*, 8 of *S. citrovorus*, 1 of *Leuconostoc mesenteroides*, 2 of *Lactobacillus brevis* and 2 of the unidentified lactobacilli from silage which were referred to in a previous paragraph. Some were tested in citrate gelatin without sugar and failed to produce either visible growth or gas.

As a diagnostic feature of the heterofermentative organisms the formation of visible gas from citrate is less reliable than gas production from glucose. All the recently isolated strains of *Streptococcus kefir* and *S. citrovorus* formed gas readily from citrate, and they did so actively in citrate milk (without any addition of glucose, yeast or plant juice) which is not the most favourable medium; but two strains of *S. kefir* which were isolated

15 years ago and the strains of *Lactobacillus brevis* have produced visible gas from citrate only slowly and erratically in any of the media in which they have been tested.

TESTS FOR THE PRODUCTION OF HYDROGEN BY LACTIC ACID BACTERIA

Hydrogen has been recorded among the fermentation products of cultures of heterofermentative lactic acid bacteria in sugar-containing media (15, 16, 6, 17), and considerable quantities of this gas have been shown to be produced from citric acid by cell suspensions of *Streptococcus paracitrovorus* (18). The possibility that the findings described in the foregoing paragraphs are partly due to the formation of hydrogen was examined by cultivating representatives of the more active and the least active gas-producing strains in **V**-tubes containing the glucose milk or glucose gelatin media. In no instance did the liberated gas contain hydrogen, for it was completely absorbed on addition of NaOH. The gas produced from citrate by heterofermentative species and enterococci was also tested for hydrogen with negative results. This evidence indicates that the diagnostic tests described in this paper rely upon the formation of CO_2 only, but the problem appeared to deserve further attention. Hassouna & Allen (19) report that *Bacterium coli* fails to liberate hydrogen in milk although it does so in lactose broth, and they suggest that the hydrogen formed in milk is used in some secondary reaction with the constituents of the medium and does not appear as gas. It seemed possible that the type of medium might similarly influence the products of the lactic acid bacteria, and two active strains of *Streptococcus kefir* were cultivated in **V**-tubes in a medium containing neither milk nor gelatin and consisting of 5% glucose, 5% peptone, 0·4% K_2HPO_4, yeastrel and cabbage juice. Again no hydrogen could be detected in the gas liberated. When the glucose was replaced by sucrose, the same result was obtained. Finally, a strain of *Bacterium coli* and a strain of *B. aerogenes* were tested in a series of media containing glucose or lactose and different sources of nitrogen (milk, nutrient gelatin or peptone) at 22, 30 and 37°C., and in every instance hydrogen was liberated and no unexpected CO_2/H_2 ratios were noted. It has thus been impossible to duplicate Hassouna & Allen's findings with *B. coli*, and attempts to demonstrate the formation of hydrogen by growing cultures of lactic acid bacteria have failed.

SUMMARY

The amount of CO_2 formed during the fermentation of sugars by the heterofermentative lactic acid bacteria depends on several factors:

(1) The sugar fermented. Gas is produced more readily from glucose than from lactose, even in the case of active lactose fermenters.

(2) The reaction and the buffering capacity of the medium. The optimum initial reaction of the medium is approximately *p*H 7 and the greater the buffering capacity the more active is the gas production.

(3) Factors which occur in plant juices and yeast. They may promote gas formation without increasing cell proliferation and apparently have a specific activating effect on the mechanism of the fermentation.

(4) The concentration of sugar, the optimum being about 5%.

The formation of CO_2 by *Bacillus licheniformis* is influenced in a similar way, but in this case the optimum initial reaction of the medium is in the region of *p*H 8–9 and the factors of (3) have little effect.

A description is given of a simple cultural test in which these organisms produce visible gas, thus differentiating them from other lactic acid bacteria or spore-forming bacilli. The media used are 5% glucose in nutrient gelatin or in a mixture of four parts milk and one part nutrient agar, with the addition where necessary of yeast extract and a plant juice. The cultures are sealed with a layer of solid agar before incubation.

The ability to produce visible gas in a citrate nutrient gelatin medium sealed with agar differentiates certain enterococci and lactobacilli from other species of the lactic acid bacteria. In the citrate medium supplemented with comparatively low concentrations of glucose the heterofermentative lactic acid bacteria produce visible gas, but certain strains fail to do so consistently.

No evidence could be obtained that growing cultures of lactic acid bacteria liberate hydrogen.

We gratefully acknowledge our indebtedness to Dr A. Cunningham for cultures of which the fermentation products had been determined quantitatively, to Dr P. M. F. Shattock and Mrs J. McNaughtan for serologically identified strains, to Dr R. St John-Brooks for type cultures, and to Mr L. J. Meanwell for strains of *Streptococcus cremoris*.

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320. COMPARISON OF THE GROWTH-PROMOTING VALUE FOR RATS OF BUTTER FAT, OF MARGARINE FAT AND OF VEGETABLE OILS

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(With 1 Figure)

Much work has been done recently in comparing the nutritive value of various oils and fats, and there are reports from several laboratories indicating that the fat of butter is superior in this respect to vegetable oils, though this opinion is by no means unanimous.

Thus Hart and his collaborators (1, 2, 3, 4) have blended 4% of different fats with skim milk and fed the mixture suitably supplemented with vitamins and mineral salts to newly weaned rats. Butter fat produced initially better gains in weight than maize oil, cottonseed oil, coconut oil, soya-bean oil or 'crisco' (a partly hydrogenated vegetable fat). After some 3 weeks the growth rates became similar for all fats. The superiority of the butter fat was considered to be due to a saturated component or components of its fatty acid fraction, and it was also shown that the unsaturated fatty acids fraction of butter could be activated by hydrogenation. Later, however, Boutwell, Geyer, Elvehjem & Hart (5) found, in the same laboratory, that the superiority of butter fat became manifest only in the presence of lactose, and that with a synthetic diet containing other carbohydrates, dextrose, sucrose, dextrin or starch, maize oil produced better growth in young rats than butter fat. More recently, Deuel, Movitt & Hallman (6) found no significant differences in the growth-promoting value, for rats, of butter fat, vegetable margarine fat and maize, cottonseed, olive, peanut and soya-bean oils when added to the extent of 32% to a diet supplemented with vitamins in which the other component was mineralized dried skim milk. Using a technique similar to that of Hart *et al.* (1, 2, 3, 4) but working with calves, Gullickson & Fountaine (7) and Gullickson, Fountaine & Fitch (8) found butter fat to be superior to tallow and lard and to a greater degree to coconut, peanut, maize, cottonseed and soya-bean oils. Harris & Rosenfeld (9) gave to newly weaned rats extracted skim milk powder mixed with 25% of olive oil, coconut oil or butter fat and supplemented with minerals and vitamins. Here again, the rats receiving butter grew better than the other animals. However, a reversal of this effect was observed by Harris & Mosher (10) with adolescent rats which grew better on a skim milk powder-coconut oil diet than on the same diet with butter fat. Freeman & Ivy (11) tested on rats an evaporated milk and a 'filled' milk in which butter fat was replaced by coconut oil, both purchased in the open market and differing also in other particulars. The rats receiving evaporated milk grew better and the difference was significant after 97 but not after 49 days. Boer & Jansen (12, 13) described experiments, of which only summaries are available in this country, in which butter, butter fatty

acids, olive oil, salad oil and margarine were added to a synthetic diet to the extent of 10%. Butter and butter fatty acids caused better growth in young rats than the other oils. Euler, Euler & Säberg(14) similarly added fats to a synthetic ration. Margarine blended from 15% soya-bean oil, 70% coconut oil and 15% hard fat proved superior to butter in promoting growth of rats.

For the last 3 years we have devoted much attention to the same problems, and the results of our experiments are reported below.

Experimental

In all experiments newly weaned (21 days old) litter-mate male rats were used. They were evenly distributed among the treatments and were housed singly. Milk or diet was offered *ad lib.* and the consumption of each rat was recorded.

(1) *Comparison of butter and margarine when incorporated in skim milk*

This experiment was carried out in August 1940. 'National butter' and 'Pool margarine' bought locally were used. Both were fed to rats by the technique of Schantz, Elvehjem & Hart(1). Some difficulty was experienced at first in emulsifying the margarine. The following procedure was finally adopted for butter and margarine: 1 pint skim milk, ½ lb. butter or margarine were emulsified with ¼ oz. gum tragacanth in a hand emulsifier. This resulted in a cream having a fat content of 20–30%. The creams were made up two or three times a week and stored at 0° C. Sufficient of each was diluted daily with skim milk to give a milk containing 4% fat. Daily fat analyses were carried out on the milks as fed. The average fat content of the 'butter' milk was 4·06% and of 'margarine' milk 3·97%.

Twelve pairs of rats were used. The milks were supplemented with Fe, Cu and Mn as described by Schantz *et al.*(1). The experiment lasted for 5 weeks; at the end of this period the animals were killed by coal gas and their body length from the tip of the nose to the centre of the anus measured according to the method of Donaldson(15). The carcasses were analysed for moisture and fatty acids as follows: the intestinal tract carefully freed from fat was removed and discarded. The carcass after drying to constant weight at 60–65° C. in an electric oven was chopped up and placed in a large Kjeldahl flask for the estimation of fatty acids by an adaptation of Leathes & Raper's(16) modification of the Liebermann saponification method.

Table 1. *Mean data for groups of twelve rats receiving fats incorporated in liquid skim milk. Exp.* 1

	Weight of rat (g.)							
Fat tested	Initial	Gain (35 days)	Carcass	Body length mm.	Milk intake ml.	g. gain/100 ml. milk intake	% moisture in carcass	% fatty acids in carcass
Butter	46·5	99·7	124·8	181·9	2033	4·91	62·8	11·5
S.E.M.*		±2·76		±1·59		±0·086		±0·59
Margarine	46·6	93·0	119·3	177·6	1886	4·92	62·0	12·4
S.E.M.		±4·18		±1·79		±0·140		±0·31

* Standard error of the mean, cf. footnote, Table 2.

The results of this experiment are shown in Fig. 1 and in Tables 1 and 2. It can be seen from Table 1 that the gains in weight made by the animals receiving butter were

Table 2. *Statistical treatment* of some of the differences observed in experiments* 1–7

Quantity studied	Difference tested	Numerical difference	Standard error of mean	*P*†	
	Experiment 1				
Gain in weight (g.)	Butter – Margarine	+ 6·7	±5·3	1 : 4	N.S.
Body length (mm.)	Butter – Margarine	+ 4·3	±1·6	1 : 7	N.S.
	Experiment 2				
Gain in weight (g.)	Arachis oil – Butter fat	+ 7·9	±5·7	1 : 5	N.S.
g. gain/100 ml. milk intake	Arachis oil – Butter fat	+ 0·65	±0·21	1 : 86	S.
	Experiment 3				
Gain in weight (g.)	Butter fat – Margarine fat	+ 7·7	±8·6	1 : 3	N.S.
	Arachis oil – Butter fat	+ 2·1	±9·2	1 : 1	N.S.
	Arachis oil – Margarine fat	+ 9·8	±6·9	1 : 5	N.S.
g. gain/g. diet intake	Margarine fat – Butter fat	+ 0·01	±0·01	1 : 2	N.S.
	Arachis oil – Butter fat	+ 0·03	±0·01	1 : 34	S.
	Arachis oil – Margarine fat	+ 0·02	±0·005	1 : 156	S.
	Experiment 4				
Gain in weight (g.)	Fraction A – Butter fat	+ 1·1	±8·9	1 : 1	N.S.
	Fraction C – Butter fat	+ 6·7	±5·2	1 : 5	N.S.
	Maize oil – Butter fat	+ 0·7	±6·8	1 : 1	N.S.
	Fraction C – Fraction A	+ 5·6	±6·4	1 : 3	N.S.
	Fraction A – Maize oil	+ 0·4	±8·2	1 : 1	N.S.
	Fraction C – Maize oil	+ 6·0	±6·2	1 : 3	N.S.
g. gain/100 ml. milk intake	Fraction A – Butter fat	+ 0·12	±0·12	1 : 3	N.S.
	Fraction C – Butter fat	+ 0·34	±0·12	1 : 61	S.
	Maize oil – Butter fat	+ 0·48	±0·10	1 : 1667	S.
	Fraction C – Fraction A	+ 0·26	±0·17	1 : 7	N.S.
	Maize oil – Fraction A	+ 0·36	±0·21	1 : 9	N.S.
	Maize oil – Fraction C	+ 0·10	±0·12	1 : 2	N.S.
	Experiment 5				
Gain in weight (g.)	Arachis oil – Butter fat	+ 1·3	±6·7	1 : 1	N.S.
	Butter fat – Cottonseed oil	+ 0·4	±5·7	1 : 1	N.S.
	Butter fat – Soya-bean oil	+ 0·3	±7·5	1 : 1	N.S.
	Arachis oil – Cottonseed oil	+ 1·7	±3·9	1 : 1	N.S.
	Arachis oil – Soya-bean oil	+ 1·6	±4·7	1 : 1	N.S.
	Cottonseed oil – Soya-bean oil	− 0·1	±3·6	1 : 1	N.S.
g. gain/100 ml. milk intake	Arachis oil – Butter fat	+ 0·36	±0·22	1 : 7	N.S.
	Cottonseed oil – Butter fat	+ 0·39	±0·22	1 : 10	N.S.
	Soya-bean oil – Butter fat	+ 0·27	±0·28	1 : 6	N.S.
	Cottonseed oil – Arachis oil	+ 0·03	±0·16	1 : 1	N.S.
	Arachis oil – Soya-bean oil	+ 0·09	±0·19	1 : 2	N.S.
	Cottonseed oil – Soya-bean oil	+ 0·12	±0·11	1 : 3	N.S.
	Experiment 6				
Gain in weight (g.)	Butter fat – Fraction A	+28·2	±4·7	1 : 5000	S.
	Butter fat – Fraction C (1943)	+ 4·1	±7·0	1 : 2	N.S.
	Butter fat – Fraction C (1942)	+12·9	±8·6	1 : 6	N.S.
	Fraction C (1943) – Fraction A	+22·7	±8·3	1 : 43	S.
	Fraction C (1942) – Fraction A	+17·8	±6·9	1 : 34	S.
	Fraction C (1943) – Fraction C (1942)	+ 8·8	±8·9	1 : 3	N.S.
g. gain/100 ml. milk intake	Butter fat – Fraction A	+ 1·25	±0·15	Over 1 : 5000	S.
	Butter fat – Fraction C (1943)	+ 0·13	±0·33	1 : 1	N.S.
	Butter fat – Fraction C (1942)	+ 0·35	±0·33	1 : 3	N.S.
	Fraction C (1943) – Fraction A	+ 1·07	±0·37	1 : 57	S.
	Fraction C (1942) – Fraction A	+ 0·87	±0·30	1 : 57	S.
	Fraction C (1943) – Fraction C (1942)	+ 0·22	±0·30	1 : 2	N.S.
	Experiment 7				
Gain in weight (g.), 6 weeks	Butter fat – Margarine fat	+ 2·6	±9·3	1 : 1	N.S.
	Butter fat – Arachis oil	+10·2	±6·3	1 : 7	N.S.
	Margarine fat – Arachis oil	+ 1·8	±6·7	1 : 1	N.S.
Gain in weight (g.), 17 weeks	Butter fat – Margarine fat	− 9·7	±8·6	1 : 4	N.S.
	Butter fat – Arachis oil	+21·6	±6·3	1 : 125	S.
	Margarine fat – Arachis oil	+23·2	±7·9	1 : 67	S.

* The statistical method used was that of the paired *t*-test of 'Student'(22, 23) as described by White(24). All comparisons were between litter-mates; the S.E.M. quoted in Tables 1, 3 and 4 were not used for this purpose.

† *P* = probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population. N.S. = not significant; S. = significant.

somewhat better than those of the rats receiving margarine, and also that the body length of the 'butter' was greater than that of the 'margarine' animals. Table 2 shows, however, that these differences are without statistical significance. The animals receiving the 'butter' milk drank more milk than the 'margarine' animals, and the gain per 100 ml. milk intake is the same for both groups (Table 1). Analysis of the carcasses for moisture and fatty acids revealed no differences between the groups. The iodine values of the body fatty acids of the 'butter' and 'margarine' animals were 56·1 and 56·4 respectively.

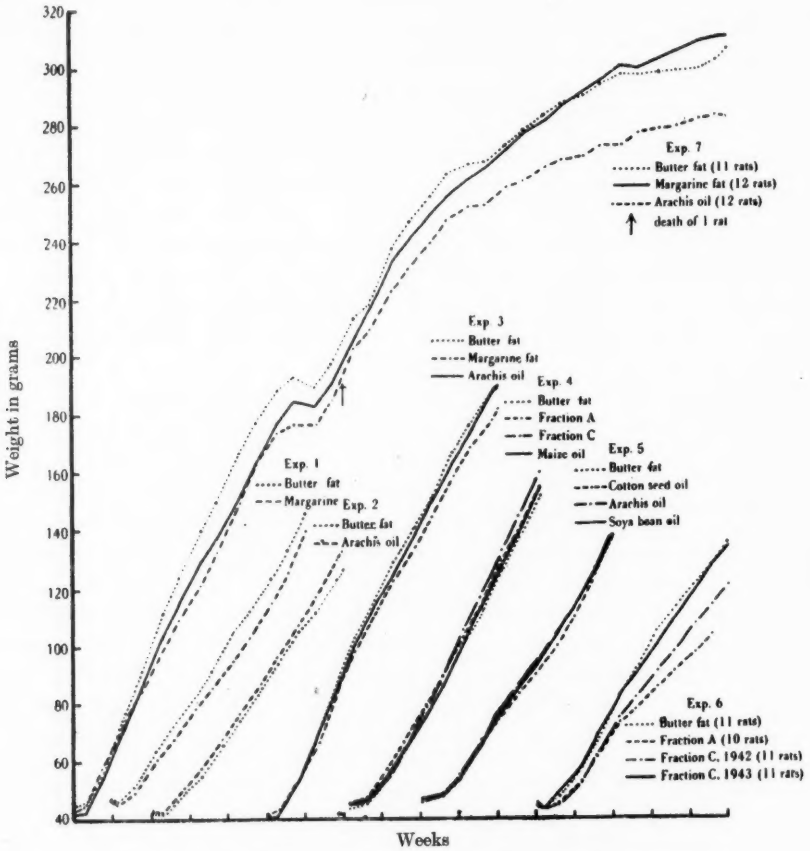

Fig. 1. Growth curves of rats in experiments 1–7.

(2) *Comparison of butter fat and of arachis oil when incorporated in skim milk*

This experiment was similar to the one described above. Samples of dried butter fat (i.v. 37·0), supplied by the Ministry of Food, and of deodorized arachis oil (i.v. 86·0) were used. The fats were homogenized into skim milk to give milks containing about 4% fat. These were made up three times a week and the bulk stored at 0° C. They were allowed to reach room temperature before being fed. The fat content of each batch was determined, the mean figures for the whole experiment were: 'butter' milk 4·37%,

'arachis-oil' milk 4·39%. Eleven pairs of rats were used and the milks were fed as in Exp. 1. In addition to the mineral supplement each rat received one drop of arachis oil containing 1 mg. of α-tocopherol and one drop of a solution of vitamins A and D once a week in the milk. The latter solution consisted of 1½ parts distilled fish-liver oil and 18½ parts of irradiated ergosterol in arachis oil and supplied 250 i.u. vitamin A and 18 i.u. vitamin D per drop. These vitamin additions were given in all subsequent experiments. The experiment lasted for 5 weeks.

Table 3. *Mean data for rats* receiving various fats incorporated in liquid skim milk. Exps.* 2, 4, 5 and 6

Fat tested	Weight of rat (g.) Initial	Gain in 32 days	Gain in 35 days	Milk intake (ml.) 32 days	35 days	g. gain/100 ml. milk intake 32 days	35 days
			Experiment 2				
Butter	43·3	—	85·5	—	1757	—	4·82
S.E.M.†			±6·16				±0·18
Arachis oil	44·4	—	93·4	—	1709	—	5·47
S.E.M.			±3·84				±0·15
			Experiment 4				
Butter	43·9	—	107·8	—	2103	—	5·12
S.E.M.			±5·64				±0·13
Fraction A	45·3	—	108·9	—	2055	—	5·24
S.E.M.			±7·16				±0·17
Fraction C	44·9	—	114·5	—	2086	—	5·50
S.E.M.			±5·33				±0·17
Maize oil	45·4	—	108·5	—	1936	—	5·60
S.E.M.			±4·52				±0·09
			Experiment 5				
Butter	45·6	—	91·0	—	1896	—	4·80
S.E.M.			±5·47				±0·17
Cottonseed oil	46·1	—	90·6	—	1727	—	5·19
S.E.M.			±6·09				±0·21
Arachis oil	46·8	—	92·3	—	1782	—	5·16
S.E.M.			±4·54				±0·13
Soya-bean oil	46·6	—	90·7	—	1746	—	5·07
S.E.M.			±7·15				±0·27
			Experiment 6				
Butter	44·6	86·4	92·5	1858	2029	4·59	4·49
S.E.M.		±6·92	±6·38			±0·22	±0·22
Fraction A	44·7	58·2	—	1717	—	3·34	—
S.E.M.		±5·02				±0·17	
Fraction C (1943)	45·5	80·9	88·4	1769	1955	4·41	4·36
S.E.M.		±10·10	±10·13			±0·39	±0·43
Fraction C (1942)	44·7	76·0	79·6	1738	1852	4·21	4·14
S.E.M.		±9·18	±9·04			±0·30	±0·36

* Particulars of the numbers of rats used in the various comparisons will be found in the text.

† Standard error of the mean, cf. footnote, Table 2.

The results are shown in Fig. 1 and in Tables 2 and 3. It will be seen that the 'arachis-oil' animals grew better than those on the 'butter-fat' milk; the difference, however, lacks statistical significance. The milk consumption of the 'butter' rats was slightly higher than that of the 'arachis' animals. The difference between the two groups is enhanced when the gain in weight per 100 ml. milk intake is calculated; moreover, this difference is statistically significant.

(3) *Comparison of butter fat, margarine fat and arachis oil when incorporated in a dry basal diet*

This experiment was carried out at the same time as Exp. 2. The same samples of butter fat and arachis oil were used; margarine fat (i.v. 45·7) was also included. The margarine used was 'special' margarine, bought locally. It was melted at 60° C. and the fat filtered off. The diets used were similar to those of Boer & Jansen (12, 13) and had the following composition: ground polished rice 70, casein (fat-free, ashless) 5, dried brewer's yeast 10, salts (de Loureiro (17)) 5, fat (butter fat, margarine fat or arachis oil) 10. Groups of eight rats were used. The experiment lasted 6 weeks.

Table 4. *Mean data for rats* receiving various fats incorporated in a dry basal diet. Experiments 3 and 7*

Fat tested		Weight of rat (g.) Initial	Gain in 6 weeks	Gain in 17 weeks	Diet intake (g.) 6 weeks	17 weeks	g. gain/g. diet intake 6 weeks	17 weeks
			Experiment 3					
Butter		42·5	146·7	—	543	—	0·27	—
S.E.M.†			±4·99				±0·012	
Margarine		42·0	139·0	—	499	—	0·28	—
S.E.M.			±9·53				±0·008	
Arachis oil		40·2	148·8	—	504	—	0·30	—
S.E.M.			±9·19				±0·006	
			Experiment 7					
Butter:	Margarine comparison	44·5	152·6	261·6	526	1638	0·29	0·16
	S.E.M.		±5·50	±5·26			±0·007	±0·008
	Arachis-oil comparison	44·5	153·6	260·4	525	1626	0·29	0·16
	S.E.M.		±5·87	±5·44			±0·016	±0·009
Margarine:	Butter comparison	43·2	150·0	271·3	489	1668	0·30	0·16
	S.E.M.		±7·46	±9·51			±0·006	±0·009
	Arachis-oil comparison	42·4	143·5	262·2	503	1632	0·29	0·16
	S.E.M.		±7·43	±8·39			±0·014	±0·005
Arachis oil:	Butter comparison	43·6	143·4	238·8	476	1556	0·30	0·15
	S.E.M.		±4·65	±2·40			±0·010	±0·012
	Margarine comparison	43·0	141·7	239·0	474	1557	0·30	0·15
	S.E.M.		±4·61	±2·18			±0·005	±0·011

* Cf. footnote, Table 3.

† Standard error of the mean, cf. footnote, Table 2.

The results (Fig. 1 and Tables 2 and 4) show that the gains made by the 'butter-fat' and 'arachis-oil' rats are very similar and are superior to those made by the 'margarine-fat' rats; the differences, however, are without statistical significance. When the gain in weight per g. diet intake is calculated the 'butter-fat' and the 'margarine-fat' diets are statistically indistinguishable, but the 'arachis-oil' diet is superior to both. These results confirm those of Exps. 1 and 2 where the fats were offered in skim milk.

(4) *Comparison of butter fat, of the solid and liquid fractions of its glycerides, and of maize oil when incorporated in skim milk*

A sample of dried butter fat (3800 g., i.v. 39·1) was separated into solid and liquid portions by the following method. The fat (in portions of about 700 g.) was dissolved in acetone (300 ml. acetone per 100 g. fat) and the solution kept at or slightly below 0° C.

in a refrigerator for a week. The solution was then filtered and the soluble fat recovered from the filtrates by removal of the acetone, first by distillation, and finally under vacuum at 100° C.; this portion (C) of the butter-fat glycerides was liquid at room temperature and contained a preponderance of unsaturated acids of the butter fat. The filtered solid fat was recrystallized from solution in acetone (500 ml. per 100 g. fat) at 0° C. for 2 days, when the deposited solids were freed from solvents by heating in a vacuum at 100° C.; this portion (A) of the butter fat was solid at room temperature and contained a preponderance of saturated acids as compared with the whole butter fat. It also retained small amounts of solid salt and curd which were removed by filtering the melted fat before the latter was used in the feeding experiments. The relatively small portion (B) of fat left in solution from the recrystallization was not used in the experiments. The separations may be summarized as follows:

	g.	% (wt.)	i.v.
Whole butter fat	3800	100·0	39·1
Fraction A (solid)	1157	30·4	19·6
Fraction B (intermediate)	373	9·8	37·7
Fraction C (liquid)	2270	59·8	47·0

Schantz, Boutwell, Elvehjem & Hart (3), in investigating the nutritive value of butter-fat fractions, mixed them with maize oil so that the resulting mixture contained them in the same proportion as the original butter. Following this procedure we incorporated the following fats at a 4% level in skim milk: (1) butter fat, (2) 3 parts fraction A + 7 parts deodorized maize oil, (3) 6 parts fraction C + 4 parts maize oil, (4) maize oil (i.v. 123). The milks were made up three times a week and the fat content of each batch determined. The mean figures for the fat content of the four milks over the whole experiment were: butter fat 4·12%, fraction A 4·19%, fraction C 4·17%, and maize oil 4·15%. The bulk of the milks was stored at 0° C.; they were allowed to reach room temperature before being fed.

Feeding tests were carried out with groups of twelve rats. The experiment lasted for 5 weeks.

The best gains were made by the animals receiving fraction C, but the small differences in gains between the groups are without statistical significance (Fig. 1, Tables 2, 3). When the gain in weight per 100 ml. milk intake is calculated, it is found that the animals receiving the less saturated fats, fraction C and maize oil made more economical use of their food than their litter-mates receiving the butter fat and the more saturated fraction A. Table 2 shows that these differences were statistically significant.

(5) *Comparison of butter fat, arachis oil, cottonseed oil and soya-bean oil when incorporated in skim milk*

Samples of dried butter fat, arachis oil, cottonseed oil and soya-bean oil having the following characteristics were used:

	i.v.	Peroxide value	Kreis value (relative)
Butter fat	39·6	1·5	0·2–0·3 red
Arachis oil	87·6	3·1	Nil
Cottonseed oil	106·4	3·4	0·2 red
Soya-bean oil	109·0	5·4	0·3–0·4 red

As in previous experiments the fats were incorporated in skim milk to the extent of 4%. The milks were made up three times a week. The mean figures for the fat percentages

of the milks over the whole experiment were: butter-fat milk 4·11%, arachis-oil milk 4·27%, cottonseed-oil milk 4·14% and soya-bean-oil milk 4·23%.

In this experiment groups of twelve rats were again used. The experiment lasted for 5 weeks.

The results are shown in Fig. 1 and Tables 2 and 3. The rate of growth was almost the same with all four milks. In gain per 100 ml. milk intake the best performance was shown by the groups receiving the arachis-oil and the cottonseed-oil milks, and the poorest by the butter-fat group. This finding is in keeping with results of the earlier experiments, where more efficient growth was made when fats of lower melting-point were given. Now, however, the differences, although suggestive, lack statistical significance (Table 2).

(6) *Comparison of butter fat, of the solid and liquid fractions of its glycerides and of the liquid fraction used in Exp.* 4 *when incorporated in skim milk*

A sample of dried butter fat was separated into solid and liquid fractions as described in Exp. 4 (p. 50). Details of the separations are as follows:

	g.	% (wt.)	i.v.
Whole butter fat	4239	100·0	37·4
Fraction A (solid)	1171	27·6	17·8
Fraction B (intermediate)	398	9·4	36·1
Fraction C (liquid)	2670	63·0	45·9

In this experiment the original butter fat and the solid (A) and liquid (C) fractions were incorporated in liquid skim milk to the extent of 4%. The two fractions were not diluted with maize oil as in Exp. 4. In that experiment the maize oil, contrary to the experience of the American workers(3), produced as good or better growth as butter fat, and it was felt that a direct comparison between butter fat and its fractions would be more profitable. Exp. 6 was started some 11 months later than Exp. 4. The remainder of the liquid butter-fat fraction used in that experiment, though stored at 36° F., had by then lost the greater part of its colour and smelt and tasted rancid. There was still enough of this fat left for a repeat test, and it was decided to find out whether its nutritive value had been affected by these changes. The characteristics of this fraction at the start of Exp. 6 were: i.v. 46·5, peroxide value 8·8 and acid value 0·35.

Eleven groups of rats were used. The experiment lasted for 5 weeks except for the group receiving fraction A for which it lasted only 32 days. The mean figures for the fat percentages of the four types of milk which were freshly prepared three times a week were: butter-fat milk 4·19, fraction A milk 4·13, fraction C (1943) milk 4·07 and fraction C (1942) milk 4·09.

The results of this experiment are shown in Fig. 1 and in Tables 2 and 3.

The animals receiving fraction A milk made the poorest gains, the best gains were made with butter fat and fraction C (1943), while fraction C (1942) occupied an intermediate position. The differences between fraction A milk and the other milks are statistically significant, but the other differences lack significance. The findings are the same when the gains in weight per 100 ml. milk intake are calculated. This experiment again shows that more saturated fats (fraction A) are less well utilized than fats of lower melting-point. At first sight the results of this experiment appear to be contrary to those

of Exp. 4, where no difference was observed between butter fat and the solid fraction A, and the liquid fraction C was better utilized than the original fat. It must be remembered, however, that in that experiment the two fractions were fed mixed with the very unsaturated maize oil, which was the fat best utilized in that experiment, and that its addition decreased the mean degree of saturation of the mixtures which were fed. The animals receiving fraction C (1942) made poorer gains than those receiving fraction C (1943). This might be taken to indicate a slight deterioration in nutritive value, but the difference is not statistically significant, and when allowance is made for differences in milk intake the utilization of these two fats was equally good.

(7) *Comparison of butter fat, margarine fat and arachis oil when incorporated in a dry basal diet*

Nicolaysen(18) found that when butter fat, cod-liver oil or margarine fat was added to a normal diet and fed to rats no differences were observed in the early stages of the experiment, but after 2–3 months the animals receiving butter fat or cod-liver oil grew better than those receiving the margarine*. Freeman & Ivy(11) also noted that differences between fats increased in experiments of longer duration. For this reason we decided to repeat Exp. 3 but to continue it for a longer period.

The basal diet used in this experiment had the following composition: ground polished rice 65, casein (fat-free, ashless) 10, dried brewer's yeast 10, salts (de Loureiro(17)) 5, fat (butter fat, margarine fat or arachis oil) 10. The fats used had the following iodine values: butter fat 36·6, margarine fat 37·3, arachis oil 88·5.

Again, groups of twelve rats were used. The experiment lasted for 17 weeks. One animal receiving the butter-fat diet died on the 13th day of the experiment, and one receiving the arachis-oil diet died during the 7th week.

It will be seen from Fig. 1 and Tables 2 and 4 that after 6 weeks the best gains had been made by the animals receiving butter fat and the poorest by those receiving arachis oil, the margarine-fat groups occupying an intermediate position, but that these differences lacked statistical significance. After 17 weeks the margarine-fat group had made slightly better gains than their litter-mates receiving butter fat, but the difference was not significant. The arachis-oil group now lagged significantly behind the other two groups. When the gains in weight per g. diet intake are calculated, the figures for the three groups become almost identical both at 6 and at 17 weeks and are obviously without statistical significance.

DISCUSSION

The series of experiments just described may be divided into two groups: in one the fats studied were dispersed in liquid skim milk, in the other they were mixed with a dry semi-synthetic diet. The results of the first group of experiments reveal in all instances only very small differences in growth. The differences in the weights of the animals as a result of comparison of any two fats are in fact statistically significant only in three out of twenty comparisons, and the slight differences observed, such as they were, are mostly in favour of an unsaturated, vegetable oil, or the less saturated fraction of butter glycerides, as compared with butter fat. When the gains in weight of the animals are considered in relation to the weight of food taken, the differences are still of a very small order, but in some instances become statistically significant. These statistically

* We have just been informed by Nicolaysen that these findings were not confirmed in a second experiment.

significant data indicate the slight superiority of arachis oil to butter fat, of arachis oil to margarine, of maize oil to butter fat and of butter fat and of its less saturated fraction C to the solid fraction A. Whilst, therefore, the differences observed are uniformly very small in magnitude, our present results afford no confirmation of the views of Hart and his co-workers (3, 4), that one or more of the higher saturated constituents of butter fat is specially favourable to the growth of young animals.

In their investigations Schantz *et al.* (3) separated the fatty acids obtained by saponification from butter by means of their lead salts, and re-esterified with glycerol the saturated and also the relatively unsaturated acids obtained. It is possible that in the course of the requisite operations the unsaturated acids might be exposed to oxidation, which might impair their dietary quality. It is to be observed that these authors state that the growth-promoting value of the unsaturated acid fraction was enhanced after the latter had been hydrogenated. Further, the esterification of fatty acids with glycerol does not as a rule furnish wholly triglycerides, the product usually containing appreciable proportions of di- and even monoglycerides. For all these reasons it seemed preferable to us to employ a method of separation of butter-fat glycerides which involved minimum handling of the latter and which also left the mixed glycerides constituted as they were in the whole butter fat. This was conveniently achieved by crystallizing the butter fat from acetone as previously described. The separation effected, although incomplete, left us with concentrates consisting respectively of glycerides containing a large preponderance of higher saturated acids (fraction A), and of glycerides containing a similar preponderance of the butter-fat unsaturated acids and characteristic short-chain saturated acids (fraction C).

The amounts of fractions A and B available did not permit us to determine their component acids or glycerides; but an indication of their general composition can be gathered from a similar separation carried out by Hilditch & Paul (19) on an English butter fat of i.v. 46·9. In this instance the solid fat obtained from solution in acetone at 0° C. was further resolved into two fractions by recrystallization from acetone at room temperature, with the result that the following three fractions were ultimately obtained:

	g.	% (wt.)	i.v.
Fraction A (hard solid)	296·1	12·1	21·5
Fraction B (solid)	601·4	24·5	36·8
Fraction C (liquid)	1553·3	63·4	55·2

The component acids and glycerides present in the whole butter fat in the combined fractions A and B, and in fraction C in the study by Hilditch & Paul (19), are shown in Table 5.

The comparison between the butter fractions of Table 5 and those employed in the present work is unfortunately more qualitative than quantitative, because the dried butter fat used by us had an iodine value of little more than that of fraction B in the investigation of Hilditch & Paul (19), whilst the whole fat in the latter had about the same iodine value as the present liquid fraction C. It is therefore probable that, in the present instance, the proportions of saturated, oleic and minor unsaturated acids would be of the following approximate order:

	Whole fat	Fraction A	Fraction C
i.v.	39·1	19·6	47·0
Saturated acids % (wt.)	60	78	53
Oleic acid % (wt.)	35	18	37
Minor unsaturated acids % (wt.)	5	4	10

The whole fat probably contained about 25% of fully saturated and about 60% of mono-unsaturated glycerides; the liquid fraction C about 18% fully saturated, about 55% mono-unsaturated group; whilst the solid portion A probably contained over 40% of fully saturated and over 50% of mono-unsaturated glycerides with very small proportions of di-unsaturated glycerides. The difference in composition between fractions A and C is amply sufficient to have demonstrated any superior growth-promoting properties of the higher saturated acids of butter fat, which are very largely concentrated in fraction A.

Table 5. *Component acids and glycerides of an English butter fat* (Hilditch & Paul(19))

	Whole fat 100% % (wt.)	Fractions A and B 36·6% % (wt.)	Fraction C 63·4% % (wt.)
Component acids:			
Butyric	3·7	1·9	4·8
Hexanoic	1·2	1·0	1·6
Octanoic	0·8	0·3	1·1
Decanoic	1·1	0·6	1·2
Lauric	2·8	1·5	1·6
Myristic	8·1	7·4	7·5
Palmitic	22·5	30·3	18·0
Stearic	11·6	21·7	8·6
Arachidic	0·9	1·4	0·5
Decenoic	0·1	0·1	0·1
Dodecenoic	0·2	0·1	0·2
Tetradecenoic	0·8	0·5	0·9
Hexadecenoic	3·0	1·6	2·4
Oleic	36·8	29·3	43·5
Octadecadienoic	5·7	2·0	5·8
C_{20-22} unsaturated	0·7	0·3	2·1
Probable component glycerides (approx.):			
Fully saturated:	% (mol.)	% (mol.)	% (mol.)
Di-C_{4-14}-monopalmitin	7	5–4	8
Di-C_{4-14}-monostearin	1	0·5–2	1
Mono-C_{4-14}-dipalmitin	0·5	0·5–2	3
Mono-C_{4-14}-palmitostearin	9	21–19	—
Dipalmitostearin	1	4	—
Palmitodistearin	0·5	1	—
Mono-unsaturated:			
'Oleo'-di-C_{4-14}	3– 9	1– 2	13
'Oleo'-C_{4-14}-palmitin	31–22	20– 7	31–33
'Oleo'-C_{4-14}-stearin	12– 6	7–17	Nil–12
'Oleo'-dipalmitin	1– 5	4–15	—
'Oleo'-palmitostearin	8–17	23–14	Nil–19
Di-unsaturated:			
Mono-C_{4-14}-di-'olein'	Nil–10	—	14–Nil
Palmitodi-'olein'	18– 4	10	22–1
Stearodi-'olein'	8– 1	3	8–Nil
Tri-unsaturated:	Nil–7	—	Nil–10

C_{4-14} = total of butyric, hexanoic, octanoic, decenoic, lauric and myristic groups.
'Oleo' = oleic plus minor unsaturated acids.

There remains the possibility that, as pointed out by Schantz *et al.*(3), the favourable results obtained by these workers with glycerides resynthesized from the 'solid' acids of butter fat, are due not to higher saturated acids but to an unsaturated acid which forms sparingly soluble lead salts. It is known that butter, and also the depot fats of oxen, contain such an acid, vaccenic acid (Bertram(20)). That this acid is responsible seems doubtful, however, since Gullickson, Fountaine & Fitch(8) found butter fat to be superior to beef tallow as a diet for calves.

The Council on Foods and Nutrition of the American Medical Association(21), in a

recent report on the relative values of butter and oleomargarine as human foods, have suggested that it would not be surprising if butter fat had a different nutritional effect, since it contains several fatty acids not present in margarine. We would point out that the only component acids present in butter which are not present in one or other of the fats usually employed in margarine, or in those used in the feeding experiments referred to in this paper either by ourselves or by previous workers, are butyric, hexoic and traces of decenoic and dodecenoic acids. Of the higher saturated acids, palmitic acid is present in fair quantity in all the vegetable fats concerned, and stearic acid is also present although usually in much smaller proportions than in butter fat; arachidic acid occurs in most of these vegetable fats in proportions of the same order as those in which it is present in butter fat. No higher saturated acid than arachidic has ever been reported in butter fat, whereas arachis oil contains small quantities of the next higher 'even-numbered' homologues, behenic and lignoceric acids.

The conclusions that butter fat is dietetically superior to fresh refined vegetable fats, and that the effective factor in this supposed superiority is a higher saturated acid present in the butter glycerides, are, therefore, not supported by our experiments.

It is perhaps permissible to wonder whether, when a slight difference favourable to butter fat or its more saturated constituents has been observed, the effect may not have been due to some inferiority in the liquid fats employed rather than to a positive effect caused by the more saturated components. We have referred above to the risk of oxidation during separation and handling of the unsaturated fatty acid fraction from butter fat. We are inclined to suggest the possibility that oxidation may have set in in some of the vegetable fats used in the earlier studies, since Schantz *et al.* (3) mention the curious circumstance that, on mixing resynthesized glycerides from the unsaturated fatty acid fraction of butter with a sample of maize oil, the mixture set to a gelatinous mass. This might possibly have been due to the presence of oxidized oil.

It was in order to test this possibility that we undertook the series of feeding trials in which a soya-bean oil of edible quality which had been stored in a partly filled vessel for a long period was compared with fresh edible cottonseed and arachis oils and with fresh butter fat. No significant difference in growth rate or in gain per 100 ml. milk intake was observed in any group of rats, and the results merely confirm the previous findings that butter fat has no advantage in this respect over refined vegetable oils of a high degree of purity. The results with the partially oxidized fraction C are also inconclusive, though they suggest an incipient effect.

With a different technique we observed in a prolonged experiment a superiority of butter fat and of margarine fat over arachis oil, though in another similar experiment of shorter duration the results were tending the other way.

Whilst, therefore, a large body of experimental evidence has been published which suggests that under certain circumstances butter fat is superior in nutritive qualities to other fats, there is an equally impressive body of evidence which either denies or reverses this finding. Whilst it is conceivable that butter fat may contain some as yet undefined factor of specific nutritive importance, it seems doubtful if this will prove to be the case. At all events it is now extremely unlikely that a saturated fatty acid has any bearing on the problem. Indeed, the latest report from the Wisconsin laboratory (5) seems to be in accord with this last statement.

Summary

1. A series of experiments with rats is described in which the growth-promoting properties of butter fat have been compared with those of the solid and liquid fractions of butter fat, with margarine and with certain vegetable oils.

2. The separation of butter fat into fractions containing a preponderance of glycerides of saturated or unsaturated acids was effected by means of crystallization from acetone.

3. The following results were obtained by incorporating fats in liquid skim milk:

(*a*) No differences were detected between the growth-promoting properties of butter and margarine or of butter fat and deodorized arachis oil; in the latter comparison the arachis oil was more economically used than the butter fat.

(*b*) No differences were found between butter fat and maize oil alone or containing the solid or liquid fractions of butter fat in the approximate proportions in which they occur in the original fat. The less saturated oils, i.e. maize oil and maize oil + the liquid butter fraction, were more economically used than butter fat and its solid fraction mixed with maize oil.

(*c*) No differences were observed between the growth-promoting values of butter fat, arachis oil, cottonseed oil or soya-bean oil. The butter fat was rather less well utilized than the less saturated oils, but the differences were not significant.

(*d*) Poorer growth was observed with the more saturated fraction of butter fat than with the original fat or the liquid fraction.

4. When butter fat, margarine fat or arachis oil was incorporated in a dry basal diet, no differences were observed in the growth-promoting value of these fats, but the arachis oil was more economically used than the other fats. This finding was not confirmed in a second experiment of longer duration in which poorer growth was observed with the arachis-oil diet, though the economy of gains was the same with all diets.

5. It is concluded from these experiments that it is unlikely that butter fat possesses superior nutritive properties to those of other fats, and that the more saturated fraction of butter fat is certainly not superior in growth-promoting value to that of the more unsaturated fraction or to more unsaturated vegetable oils.

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ADDENDUM

Since the above was written, we have noted two further communications(25, 26) by Beznák, Beznák & Hajdu, who record that butter, horse fat, sunflower-seed oil and soya-bean oil give good growth in rats and are somewhat superior in this respect to margarine, bone fat, or linseed oil. Rape-seed oil, steam-heated or hydrogenated, was also only slightly inferior to butter fat, but rape-seed oil, refined with sulphuric acid,

gave very poor results; this, doubtless correctly, was attributed to the presence of a toxic substance in the oil.

Beznák *et al.*(25) attribute their good results with butter to the content of C_6–C_{14} saturated acids in the latter, stating that their views are in agreement with those of Schantz *et al.*(1, 2, 3). It must be pointed out first, that this is not the case, since the latter authors believed a saturated acid of high molecular weight (not the C_6–C_{14} acids) to be responsible for the supposed superiority of butter; and, secondly, that horse fat, sunflower-seed oil and soya-bean oil (which gave results almost equal to those with butter) contain no acids with less than 14 carbon atoms, and only minor proportions of myristic acid.

In our opinion Beznák *et al.*(25, 26) have failed to produce any evidence in support of the explanation which they offer. The observed differences (as admitted in the instance of the acid-refined rape oil) appear more likely to be due to contaminants or oxidation products of the oils producing deleterious effects on rate of growth rather than to any superiority *qua* glyceride components of the fats which led to satisfactory growth of the animals.

The full report of the work of Deuel *et al.*(6) has now appeared(27, 28, 29, 30), while Boutwell, Geyer, Elvehjem & Hart(31) have published a paper disagreeing with the views of the above authors. Finally Euler, Euler & Säberg(32) have confirmed their earlier views(14) and are of the opinion that there is no evidence of any special nutritive factors in butter fat.

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ERRATUM

J. Dairy Res. **14** (1 & 2), p. 59

Table 1: column 2, delete 'Yellow units'.
column 3, delete 'Yellow and red units'.

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321. REACTIONS AND PROPERTIES OF ANNATTO AS A CHEESE COLOUR. PART II

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In Part I of this work (1) dealing with the standardization of annatto for cheese colouring, the conclusion was drawn that the colour of a dilute alkaline solution of annatto, as measured by the Lovibond tintometer, gave a reasonably accurate estimate of its dyeing properties in cheese curd. This conclusion has been sharply criticized by Carrie (2, 3), who maintained that an acid medium, simulating conditions in cheese, was necessary for an accurate estimate, and suggested the use of a series of coloured plates as a standard of comparison for the colour of cheese. This latter method can, however, hardly be regarded as a practical procedure for the standardization of annatto extracts.

Comparison of the Pulfrich gradation photometer and Lovibond tintometer for the standardization of annatto solutions

Carrie expressed the view that the Lovibond tintometer did not give a sufficiently accurate comparison, citing the work of Gibson & Harris (4), who, in their study of Lovibond glasses, drew attention to the inaccuracies caused by losses of reflected light. To check this point, estimates of the colour strength of several different samples from four brands of commercial annatto extracts have been made with the Pulfrich gradation photometer and the Lovibond tintometer. The results are given in Table 1.

Table 1. *Relative colour strengths of annatto extracts**

Annatto sample no.	Photometer (S 47 filter) (alkaline solution) (*p*H 5·5)		Tintometer (alkaline solution)	
	Yellow units	Yellow and red units	Yellow units	Yellow and red units
1	1·00	1·00	1·00	1·00
2	1·13	1·03	1·16	1·16
3	1·07	1·22	1·16	1·11
4	1·07	1·07	1·06	1·06
5	1·03	1·10	1·11	1·05
6	0·98	1·10	0·97	0·93
7	0·83	0·90	0·89	0·90
8	0·81	0·81	0·78	0·80
9	0·52	0·84	0·55	0·57
10	0·42	0·71	0·47	0·49
11	0·42	0·66	0·45	0·49
12	0·26	0·44	0·23	0·21
13	0·06	0·27	0·06	0·06
Average	0·74	0·85	0·76	0·75

* Results for a representative sample (no. 1) used in the manufacture of many of the cheeses made for this investigation are taken as unity.

For comparing the results in Table 1, the Pulfrich photometer readings for the annattos in alkaline solution may be taken as standard. On this basis the average value for the photometer reading at *p*H 5·5 was 15% higher than the standard, but the individual

samples showed a variation from 9% below to 350% above, the greatest divergence being noted for the weaker samples. At *p*H 5·5, there was some formation of colloidal substances, and the comparisons were upset by the resulting decreased transmission of light through the samples.

With the tintometer, and considering yellow units only, the values for annattos in alkaline solution averaged 3% higher than the standard, ranging from 11% below to 12% above. Taking the yellow + red units, the values were, on the average, only 1% higher than the standard, but the individual results ranged from 19% below to 17% above. It is evident, therefore, that with dilute alkaline solutions of annatto, the values obtained with the tintometer agree well with those found by the Pulfrich photometer. The tintometer can therefore be regarded as a satisfactory instrument for estimating the colour strength of annatto.

Chemical and biochemical changes

In Part I it was concluded, on the basis of trials made in solution, that certain enzymes were not responsible for causing discoloration of annatto in cheese. Carrie expressed the view that the trials should have been carried out with cheese rather than in solutions(2). In examining the effect of the enzymes catalase, peroxidase, oxidase and dehydrogenase—work done in connexion with studies on an outbreak of discoloration in cheese—conditions were naturally chosen in which the reaction, temperature and substrate were suitable for the enzymes tested. Moreover, the findings of Part I are amply confirmed in practice. Raw-milk cheese, containing oxidase, peroxidase, dehydrogenase and a variety of other enzymes, does not normally discolour, nor does cheese made with heavy infections of mastitis milk, containing catalase, develop this defect.

Annatto in the cheese-making process—relationship of strength of annatto to colour of cheese

Carrie has disputed the contention that there is 'a proportionality between the colour of cheese and the initial concentration of annatto in the milk'. The results cited in Part I admittedly did not show an accurate correlation. This matter has therefore been subjected to a more detailed examination. A sample of annatto was used in three vats of milk on each of 4 days. The first of the three vats of milk was made into cheese without the addition of cheese colour. For the second vat, the normal amount of colour was used, and, for the third, on alternate days, half the normal or twice the normal amount was added. The results are given in Table 2. In Table 3 they are shown corrected for the colour of the cheese to which no annatto was added.

From these results the following observations are of particular interest:

(*a*) The colour of mature uncoloured cheese (6 months old) made from mixed-herd milk on four consecutive days averaged:

Yellow units	4·7 (4·3–4·9)
Yellow + red units	6·4 (5·7–6·8)

(*b*) Cheese made under the same conditions as in (*a*), but with the usual proportion of annatto, averaged:

Yellow units	8·8 (8·4– 9·5)
Yellow + red units	12·3 (11·8–13·4)

(*c*) Cheese made under the same conditions as in (*a*), but with double the usual proportion of annatto, averaged:

Yellow units	13·0 (12·6–13·8)
Yellow + red units	17·8 (17·1–18·6)

(*d*) The increase in colour in mature cheese due to the addition of annatto averaged:

	Yellow units	Yellow + red units
Half usual amount of annatto	3·1	4·1
Usual amount of annatto	4·1	5·9
Twice usual amount of annatto	8·3	11·4

Table 2. *Relationship between amount of colour added and colour of final cheese*

Manufacturing day	Proportion of annatto used	Cheese: 1 month old Y.*	1 month old R.	1 month old Y. + R.	3½ months old Y.	3½ months old R.	3½ months old Y. + R.	6 months old Y.	6 months old R.	6 months old Y. + R.
1	Nil	4·5	1·5	6·0	5·1	3·0	8·1	4·3	1·4	5·7
	Usual†	8·6	3·6	12·2	9·8	4·0	13·8	8·4	3·4	11·8
	Half usual	7·3	3·4	10·7	7·8	3·2	11·0	7·4	2·4	9·8
2	Nil	4·2	1·2	5·4	4·5	1·8	6·3	4·7	1·8	6·5
	Usual	7·4	3·0	10·4	7·7	4·0	11·7	8·5	3·7	12·2
	Twice usual	12·5	3·9	16·4	12·0	4·9	16·9	13·8	4·8	18·6
3	Nil	4·4	1·5	5·9	4·5	1·6	6·1	4·8	1·8	6·6
	Usual	7·5	3·7	11·2	8·6	3·4	12·0	9·5	3·9	13·4
	Twice usual	12·6	5·0	17·6	11·1	4·3	15·4	12·6	5·0	17·6
4	Nil	4·5	1·4	5·9	4·5	1·8	6·6	4·9	1·9	6·8
	Usual	8·5	3·7	12·2	8·8	3·3	12·1	8·9	3·1	12·0
	Twice usual	12·0	5·0	17·0	13·5	4·6	18·1	12·6	4·5	17·1

* Neutral tints varied from 0·1 to 0·3. † 4·3 ml. per 100 lb. of milk.

Table 3. *Coloured cheese—increase in yellow + red units over uncoloured cheese*

Manufacturing day	Proportion of annatto added	1 month old	3½ months old	6 months old
1	Usual	6·2	5·7	6·1
	Half usual	4·7	2·9	4·1
2	Usual	5·0	5·4	5·7
	Twice usual	11·0	10·6	12·1
3	Usual	5·3	5·9	6·8
	Twice usual	11·7	9·3	11·0
4	Usual	6·3	5·5	5·2
	Twice usual	11·1	11·5	10·3

This work shows that there is a proportionality between the amount of annatto added and the colour of mature cheese, and that cheese tend to increase in colour on keeping. Measurement of the colour of normal cheese made with the usual and twice usual proportions of colour show a variation of about 10%, and this variation is of the same order as for the measurement of colour in 'uncoloured' cheese.

STANDARDIZATION OF ANNATTO SOLUTIONS FOR CHEESE COLOURING

Carrie (2, 3) considered it reasonable to assume that the standardization of annatto should be carried out in solution at about the *p*H of cheese (5·0). Under these conditions, however, the annatto is present partly in the colloidal state ((1) and Table 1), rendering colorimetric assay by the tintometer, and especially by the photometer, difficult. Table 1 shows that the correlation between the colour of annatto solutions determined at *p*H 5·5

is not so close as that obtained in alkaline solution, but in order to make a practical test, about seventy vats of cheese were made (during two seasons, using several different samples of four brands of annatto, from British and foreign sources, standardized both in acid and alkaline solution. On many occasions, 10% more or 10% less dye than used for the control was added to one vat, in order to find whether such variations (i.e. about the same order as the variations of the tintometric measurements) were discernible in the finished cheese. The cheese, made under standard factory conditions, were examined at intervals up to 6 months and proved to be normal. They were submitted for criticism to several experienced cheese-makers and it was found that:

(*a*) With the exception of cheese coloured with one unusual sample of annatto of foreign origin,* mature cheese coloured with annattos from various sources, standardized in alkaline solution prior to manufacture, showed no greater variations in colour than the day-to-day differences between controls (i.e. about $\pm 10\%$).

(*b*) Variations of $\pm 10\%$ in the amount of annatto added were not detectable in cheese several weeks or more old, but 20% variations were always discernible.

(*c*) Mature cheese coloured with various annatto extracts standardized in alkaline solution were more uniform in colour than those coloured with extracts standardized in solution at *p*H 5·5.

Carrie subsequently altered his opinion with regard to standardizing annatto colour in acid solution and now agrees with these findings(5). He states: 'of the 15 samples of colour tried...the alkaline strength gave a truer estimate of the cheese-dyeing value in 9 cases, the acid strength in 2 cases, while in 4 cases the differences were not significant.... The evidence was definitely in favour of the alkaline strength....'

Keeping quality of commercial annatto extracts

The tests cited in the previous paper have been repeated, using as containers different types of glass, and also stainless steel (as recommended by Carrie). The latter proved to be superior to glass for storage of annatto extract under air (at 30° C.). However, there was no loss of colour in samples stored in glassware under vacuum for 3 months.

Discoloration of annatto in cheese

In Part I of this work it was submitted that 'pink discoloration is produced merely by the removal of some of the yellow component of the pigment'. Carrie(2) considered that this implied a belief that 'annatto consists of two components whose colours correspond to the red and yellow of the Lovibond scale respectively'. On reflexion, however, it appears that the statement is reasonable, since it was shown that (*a*) annatto acts as an indicator, being reddish in acid solution, (*b*) it tends to precipitate in acid solution, (*c*) the acid-precipitated pigment on the casein of cheese is more red than the same strength dye in alkaline solution, and (*d*) oxidation preferentially destroys the more yellowish (unprecipitated) fraction, leaving the more red precipitated dye, which gives a very pink appearance in cheese (or on filter paper) and which gives a considerable increase in the ratio of red : red + yellow units of the Lovibond tintometer. (Before making these observations, we believed that the pink colour found in this type of cheese

* This was an old sample, but did not show the usual precipitate and in other respects was unusual. The cheese were of about the usual depth of colour, but yellow rather than orange in tint.

discoloration was caused by extra pigmentation rather than by the differential decolorizing process described.)

DISTRIBUTION OF ANNATTO BETWEEN CHEESE AND WHEY

The experiment described in Part I has been repeated, using an improved technique, the annatto retained in the whey being extracted after making alkaline with ammonia and adding alcohol to precipitate the protein. The clear filtrate was compared with similarly treated annatto at the same dilution as that used in the cheese vat.

Three different brands of annatto were used on four consecutive days. The results are shown in Table 4.

Table 4. *Retention of annatto by whey*

Annatto brand	Amount added	% pigment added to vat retained in whey	$K = \frac{\text{Pigment retained in whey}}{\text{Pigment retained in cheese}}$
A	Nil	—	—
	Usual	15	0·18
	Half usual	14	0·16
A	Nil	—	—
	Usual	16	0·19
	Twice usual	16	0·19
B	Nil	—	—
	Usual	17	0·20
	Twice usual	18	0·22
C	Nil	—	—
	Usual	12	0·14
	Twice usual	11	0·12

The distribution of the dye between whey and cheese is apparently constant for any one sample of annatto. The proportions found are actually rather higher than those reported earlier (1).

SUMMARY

Further work on the standardization of annatto for cheese colouring supports the author's earlier conclusions (1), viz.

(1) The colouring power of annatto extracts in cheese can be estimated by their tinctorial power in dilute alkaline solution.

(2) A simple tintometer of the 'Limitester' type is suitable for this work, a standard orange disk, or Lovibond yellow and orange glasses being used as the standard.

I am grateful to Dr F. H. McDowall for helpful criticism, and to the Institute Factory staff for the manufacture and grading of the cheese.

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322. BACTERIOPHAGE INFECTION IN CHEESE MANUFACTURE

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(With 5 Figures)

The preparation of starter cultures and the control of their growth in the cheese vat are the most important factors in successful production of high quality Cheddar cheese. This control is of special importance for experimental work on the manufacturing process, since regular acid development, giving reproducible results from day to day, is necessary before the effects of other variations in technique can be evaluated. In commercial practice the elimination of irregularities in starter action would go further than any other factor in converting cheese-making from an art to a science.

Hammer & Babel(1) have recently given a very comprehensive review of work done on starter cultures in the past. It is therefore unnecessary for us to cover the subject again, but we would like to clarify the somewhat confusing impression given in the review of work on bacteriophage done at this Institute. The confusion arises because the authors do not relate later work to earlier work. It is, for instance, misleading to refer to Whitehead & Hunter's earlier hypotheses on the effect of aeration; on the effect of size of inoculum; on the origin of bacteriophage within cultures, without indicating that the later work showed the falsity of these hypotheses and replaced them with a sounder hypothesis, viz. that phage is frequently air-borne and hence very readily infects cultures under conditions which had previously been thought to preclude infection. We hope in the following paper to make our present viewpoint clear and to give new facts which have emerged in the last 2 years.

Our work has been confined almost entirely to one aspect of the starter question, viz. control of the acid-producing activity of the streptococcal cultures used in cheese manufacture. In recent years this problem has resolved itself essentially into the elimination of bacteriophage infection.

This paper is divided into the following main sections:

Section I. A review of the general trend of our past work on bacteriophage infection.

Section II. A consideration of the various ways in which a cheese vat may become infected with phage.

Section III. The practical measures being tried in New Zealand in attempts to prevent phage infection of starter cultures.

Section I

(i) *Slow acid development in cheese vats*

Various investigators have shown that there are several possible causes for slow acid development during the cheese-making process:

(i) Abnormal milk from cows suffering from mastitis(2).

(ii) Milk rendered abnormal owing to the growth in it of specific bacteria(3).

(iii) Starter cultures which are unduly injured by exposure to the temperatures used during the 'cooking' ('scalding') process (4).

(iv) Lysis of the starter organisms by bacteriophage (5).

We have found that bacteriophage infection is the most frequent cause of slow acid development in commercial practice, especially where the slowness or stoppage is of sudden occurrence. Trouble of this type is readily distinguished from slowness due to cause (ii) where the hold-up in acid production occurs in the early stages of the vat process; there is usually a measure of recovery later on because the bacteria are merely hampered in their growth, not permanently damaged. Slowness due to causes (iii) and (iv), however, is the result of damage or destruction of the starter bacteria; hence the cheese-making process starts normally and acid development becomes slower (or stops entirely) owing either to heat damage or complete lysis of the bacteria. We have no experience of slowness due to cause (i).

In New Zealand commercial cheese factories these types of trouble give a much clearer picture than they do in some other countries because (i) the milk is pasteurized, (ii) there is a widespread use of starters consisting of a single strain of streptococci. In countries where the cheese is made from raw milk and where starters containing a mixture of several strains of streptococci are used, the picture is necessarily confused because the organisms concerned in acid production come from the milk as well as from the starter, and the various strains are not all equally affected by the adverse influences indicated above; in particular, any phages which may be present are not likely to be active against all the strains of streptococci. Marked slowness or complete stoppage in acid development is therefore a much less frequent occurrence than under New Zealand conditions. Although this might at first sight appear to be an advantage from the cheese-maker's point of view, it is coupled with the very serious disadvantage, from the standpoint of cheese quality, of irregularity in acid production from day to day. The raw milk, unless of very high quality, contains acid-producing bacteria which vary in numbers and activity from day to day and the 'mixed' starter culture, with time, suffers changes in the balance of the various acid-producers present in it.

It became quite clear in the early stages of work on starters at this Institute that the best hope of attaining regularity in acid production in the cheese vat lay in the use of milk as free from bacteria as possible and starter cultures consisting of single strains of streptococci. The rapid extension of the use of 'single-strain' starters in the commercial factories of New Zealand indicates that they have proved in practice to have advantages, one of which is regularity of action. All our investigations in recent years have therefore been based on single-strain cultures.

(ii) *Air-borne bacteriophage as the most insidious form of infection*

As it gradually became evident that bacteriophage was the main cause of failure of the 'single-strain' cultures of streptococci in use as cheese starters, the problem narrowed itself to a search for methods to eliminate the effects of phage on the starter organisms during the cheese-making process. Experience, both in commercial factories and in the laboratory, seemed to suggest at first sight that phage specific to a given strain of streptococcus originated spontaneously in a culture of the organism under the influence of certain environmental factors, viz. aeration of the milk medium, small size of inoculum, and use of presumably abnormal milk as a medium. But these postulated causes of

the appearance of phage within a culture proved to have no basis in fact, when it was subsequently discovered(6) that air-borne phage was usually prevalent wherever the particular organism concerned had been propagated in large quantity for some time (e.g. in cheese vats). It immediately became evident that aeration led to infection with the air-borne phage; that a small inoculum allowed the contaminant phage to show its effect more quickly; that 'abnormal' milk was merely milk which happened to be used in a place where there was a higher-than-average concentration of air-borne phage. In brief, recognition of the existence of air-borne phage enabled a single explanation of all the phenomena to be offered. Contamination with air-borne phage accounted for the mysterious failures of cultures which still occurred in dairy factories in spite of the adoption of an aseptic bacteriological technique.

(iii) *Origin of air-borne bacteriophage*

After five or six years' observation of the behaviour of 'single-strain' starters in commercial cheese factories we find it possible to visualize a method by which phage infection arises, slowly develops and finally becomes widespread in the atmosphere. Starting with the hypothesis that phage is a submicroscopic organism, we may suppose that the various phage races are as widespread in nature as bacteria in general. They lie dormant in dust for long periods, since each phage race is relatively specific and can multiply only at the expense of a few particular strains of streptococci. The cheese vat, open to the air, is a large bacterial culture vessel into which a particle of the appropriate phage hitherto dormant ultimately finds its way by chance. Multiplication of the phage occurs during the cheese-making process, and since the whey is inevitably splashed about during the vat process, is passed through a high-speed separator and is then transported round the countryside for pig feeding by the farmers, phage is necessarily broadcast in a very short time. It then becomes difficult in the absence of strict precautions to prevent infection and lysis of cultures of the particular strain of streptococci in that environment. We have observed the sequence of events on many occasions. A fresh strain of streptococci when first put into use, even with the crudest of handling, is at first trouble free during the period when no phage can be demonstrated in the whey in the vats. Soon after the phage appears in the whey there are occasional failures where (in the absence of special precautions) the starter culture is not clotted and is obviously useless for cheese-making purposes. A fresh supply of the same culture from the laboratory may be satisfactory for a few days, but the stage is soon reached where the starter cannot be relied upon at all. Cases have occurred where fresh culture from the laboratory failed regularly to clot the bulk starter milk in the factory.

The trouble-free period before phage appears in the whey varies considerably in extent both with the culture used and with the location of the factory. In the Taranaki province of New Zealand, where there are about one hundred cheese factories, one of the first single-strain starters put into use (HP) gave no trouble in several factories for about six months, although at that time an aseptic technique in handling starter culture was not employed. When, however, failures of starter due to phage infection occurred, it was not long before HP culture became known to cheese-makers throughout the province as a very 'delicate' or 'dangerous' starter. On the other hand, in the Marlborough province (of the same order of size as Taranaki), where there are only five small factories, culture HP gave little trouble for about two years. Even after that period the factories got into

difficulty only one by one over quite a long period. Eventually, however, all the factories had the same difficulty in maintaining HP in the absence of special precautions. It seemed clear that the small size and isolated situations of the factories in Marlborough resulted in a much more gradual spread of phage for culture HP throughout the district.

(iv) *Cure of starter trouble in the past and its relation to present-day knowledge*

Before phage infection of starter cultures was recognized the cheese factory manager's first reaction to starter trouble was to change the starter. This applied both with 'single-strain' and 'mixed' cultures. He usually tried to get a culture from a factory some considerable distance away. Translated in the light of our knowledge on phage infection this means that a change was made to a culture for which there were no phages at the moment in the environment. If in any given case slow acid development in cheese vats is due to phage infection of the starter organisms, a change of culture is therefore a logical step *provided* the organisms in the new culture are immune to attack by the phages at present widespread in the factory. But the relief gained is only temporary, and its duration depends on the time taken for a particle of phage active against the new culture to find its way by chance into a vat. Anderson & Meanwell(7) in England work deliberately on these lines. They change the starter culture in use as soon as any appreciable concentration of phage active against it can be detected in the cheese whey. The disadvantage of the system in our opinion is that it involves the use of several different cultures for cheese manufacture, no two of the cultures having exactly the same acid-producing characteristics. The cheese-maker has to alter his manufacturing procedure with each culture. So long as there is any hope of gaining full control over the activity of any one culture so that it can be used day after day for an indefinite period, in spite of the presence of phage, it is better in our opinion to persist in the attempt to discover the appropriate method. A system of rotation in the use of starter cultures may, however, prove necessary if it is found impracticable in any given case to eliminate phage infection.

SECTION II

(i) *The various channels by which cheese milk becomes infected with phage*

Trouble due to phage in cheese manufacture is not confined to direct infection of the mother culture and bulk starter during their preparation. Infection in the cheese vat can occur from sources other than the starter itself. The complete picture can be set out as follows:

(*a*) Phage falls into cheese vats as a dust or droplet infection. It multiplies during the 'cooking' ('scalding') process and is thus present in the whey in a concentration the extent of which depends upon the amount of the original infection. As a result, the vats and all utensils are contaminated with phage, which if not destroyed by a sterilizing process may be present next day in an amount sufficient to cause lysis of the starter organisms during the making process.

(*b*) The whey separator (used to extract the trace of fat present in whey), in treating a highly infected whey, spreads a droplet infection through the atmosphere. All other splashing of whey (e.g. from vat taps) contributes to this infection. The droplets settle throughout the night in the cheese vat and on utensils and may of themselves, or

combined with the infection described under (*a*), give enough phage to lyse the starter organisms in the cheese vats.

Further, the air-borne droplets may, in the absence of adequate precautions, infect the bulk starter or mother culture so that the culture either fails to clot or, although clotted, carries enough phage with it into the vat next day to cause its own destruction during the course of cheese manufacture.

(*c*) The farmers take home whey for the feeding of pigs. In New Zealand this whey is commonly carried in the cans in which the milk was delivered to the factory. There are usually no facilities for complete sterilization of cans on the farm and, unless the washing process is very thorough, sufficient phage may remain in the cans to give a dangerous concentration in the milk the next day. The flash pasteurization of milk as practised in New Zealand factories (150–160° F. for about 30 sec.) is not effective in destroying phage. Hence phage present in the milk received on any given day may cause trouble in certain vats, or alternatively phage in the milk plus phage from sources described under (*a*) and (*b*) may in certain vats make an aggregate big enough to cause either partial or complete lysis of the starter during the process of manufacture.

(*d*) The bulk starter culture itself (assuming that the mother culture is uninfected) may become infected with phage either through improper handling or through air-borne droplet infection in the absence of special precautions. If the infection is heavy enough, the streptococci are lysed in the starter culture itself which then does not coagulate normally. This is immediately obvious to the manager and the starter is not used in the vat. The more insidious trouble occurs where a mother culture free from infection is used to inoculate a bulk culture milk which has become slightly infected during the cooling process after sterilization. If the infection is not sufficiently heavy to cause lysis in the bulk culture, the starter appears normal the next morning, although some multiplication of phage has taken place before the starter coagulated. When such a culture is added to the milk in the cheese vat, even in the absence of phage from sources described under (*a*), (*b*) and (*c*), the starter organisms are lysed at some stage in the process by the phage present in the bulk culture itself. The extent of the original infection of the culture determines whether stoppage in acid development occurs early or late in the process.

Since slowness or stoppage in acid development may be due to one, two, three or all four of the above causes, it is readily to be understood that a complicated picture is sometimes presented in commercial factories. There is liable to be still more confusion when the cheese-maker is using a 'mixed' starter culture containing several strains of streptococci, only some of which are lysed by the phage or phages present.

(ii) *Relative importance of the various factors*

In our experience complete failure in acid development in the early stages of the cheese-making process (say up to the time the whey is removed from the vat) is almost always due mainly to phage infection in the bulk starter itself. Infection from the vat and utensils, from air-borne droplets, and from phage in the milk is definitely on a smaller scale because there has been no opportunity in these instances for the phage to multiply overnight, whereas in the starter culture the phage has been actively developing on its host organism. Infection from sources other than the starter itself seems to give 'sluggish' vats in which acid development tends to become slow towards the end of the process. We suspect that many 'normal' vats suffer from a slight slowing in acid

development (due to phage infection) which goes unnoticed because there is no 'control' vat completely free from infection.

(iii) *The cycle of infection*

A consideration of the way in which phage infection develops and spreads will indicate that trouble due to phage can develop into a cycle. An abnormally slow vat (whatever the original source of the phage) yields a large volume of whey in which the concentration of phage may be ten to one hundred times greater than hitherto. The infection in the traces of whey left on vats, cans, and equipment is automatically increased ten- to a hundred-fold unless cleansing and sterilizing operations are more thorough. The air-borne droplet infection is also increased. Hence there is a greater risk of starter failures on subsequent days, and repeated failures in the vats still further increase phage infection in the environment. The cycle can be broken either by thorough cleaning and sterilization of all equipment or by changing the starter culture to one not susceptible to the same phage, as has already been discussed. Fig. 1 illustrates the cycle of infection.

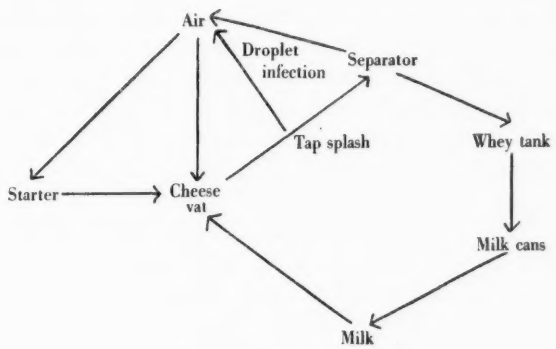

Fig. 1.

(iv) *Constant phage infection in cheese vats*

The impossibility in commercial practice of destroying every trace of phage each day does not appear to be a serious matter. So long as the amount of phage in a vat is not sufficient to affect the acid-producing power of the starter before the vat operations are finished, the presence of phage in the whey is of significance only in so far as it provides the source of phage contamination for subsequent days. There are many commercial factories in New Zealand using the same 'single-strain' starters day after day for the whole season. Phage is constantly present in the whey, but the precautions adopted keep the concentration below that necessary to cause slowness in acid production; the starter is safeguarded against air-borne infection by appropriate methods to be discussed later. The quality of the cheese does not suffer at all.

(v) *Methods for investigation of the cause of slow acid development*

During the past two years it has been necessary to devise methods for determining in any given case of 'slow' vats whether the trouble is due to phage contamination of the starter itself, of the vats and equipment in the factory, or of the farmer's milk. All three

types of trouble have been identified, infection of the starter being by far the most frequent. The following methods of investigation have been used.

(*a*) *Detection of phage contamination in starter*

Bulk starter culture is usually prepared in New Zealand in 20 or 25 gal. cylindrical cans. Mother cultures are prepared in conical glass flasks. During the past ten years it has become almost standard practice for factory managers to treat the mother culture in exactly the same way as it is treated in a bacteriological laboratory; the flasks are plugged with cotton-wool and inoculation is performed with glass pipettes sterilized in a metal cylinder by dry heat. The mother cultures are usually prepared (or at any rate inoculated) away from the main factory building. Thus there is usually little or no trouble experienced in maintaining the mother cultures free from any kind of infection. Phage infection occurs more frequently, however, in the bulk starter which is more difficult to protect from air-borne contamination. Quite frequently, where some rather makeshift method of protecting the bulk starter is used, the culture appears quite normal as it is put into the vats, but it is, nevertheless, slightly infected and the vats become 'slow' during the process. There are two methods for checking the condition of the bulk starter:

(i) A sample is taken first thing in the morning with a sterile 1 ml. pipette. Three drops from the end of the pipette are put into a culture tube containing 10 ml. of sterilized skim milk. The tube is incubated at 22°C. If the skim milk fails to clot within 24 hr., it is presumptive evidence that the starter in the can was infected with phage. If the milk clots in normal fashion, it is presumptive evidence that any slowness which may have occurred in the vat was not due to phage from the starter.

(ii) A sample is taken and a tube of milk inoculated as in the first method, but the tube is incubated at 37°C. instead of 22°C. Slides are prepared from the sample at intervals; they are stained and examined under the microscope. It can thus be determined positively whether lysis of the organism occurs within 6 or 7 hr. (the period during which the starter is required to produce acid in the cheese vat).

Neither of these methods is of use as a routine test to the cheese-maker because the starter must be used in the cheese vat before the result of the test is available. Their purpose is to decide whether a slowness in the vat due to phage is the result of starter infection or infection of the cheese milk from other sources.

(*b*) *Detection of phage contamination from factory and farm equipment*

Samples of milk taken from farmers' cans or within the factory at any stage during its passage into the vat can be examined for the presence of a specific phage provided the culture susceptible to that phage is available.

It will again be evident here that in all work involving detection of phage infection it is difficult to get a clear picture unless 'single-strain' starters are used. This follows from the relative strain specificity of most streptococcal phages.

Samples of 10 or 15 ml. are collected in sterile plugged tubes. Samples of raw milk are pasteurized at 155°F. for 10 min. to destroy organisms which would confuse the picture under the microscope later. This heat treatment does not materially weaken any phage which may be present. Samples of pasteurized milk need no further heat treatment. When all the samples have been collected, they are inoculated with three drops of an uninfected culture of the appropriate starter culture (a control culture in sterilized milk

should be put up). All the inoculated samples are incubated at 37° C., and slides made at intervals are stained and examined under the microscope. In samples which contain phage there is gradually increasing growth followed by lysis (indicated by partial or complete disintegration of the organisms). This test provides a means for detecting milk supplies which contain a significant amount of phage or pieces of equipment after contact with which the milk is more heavily infected with phage than before.

It is necessary to use discretion in interpreting the results of the test. Where a certain starter culture has been in use in a commercial factory for a considerable time there are liable to be traces of phage in all milk samples taken in the factory. The aim of the test is to determine whether in any of the samples there is sufficient phage to lyse the starter organisms within 6 or 7 hr. Such milk present in sufficient amount in a vat will cause slowness.

The cure for trouble in the cheese-making process due to phage infection from equipment is daily sterilization by heat or a disinfectant such as hypochlorite (which is very effective in destroying phage). This is straightforward, although difficult to have applied consistently from day to day in so far as milk cans on the farms are concerned. It is not necessary to discuss this side of the matter further.

The elimination of phage infection in the starter, by far the most serious cause of trouble, is much more difficult. It involves complete protection of the starter from all kinds of infection throughout the whole process of milk heating, cooling, inoculation and incubation. The succeeding section of the paper deals with the means which have been adopted to attain this end in commercial practice.

SECTION III

(i) *The power of the air-borne infection and extent of its spread*

Recognition of the presence of air-borne phage in the surroundings of cheese factories (6) was a turning point in research on maintenance of starter cultures. The presence of phage in the atmosphere was first demonstrated by various methods of collection (6): (1) aspiration of the air into sterile water or through sterile cotton-wool; (2) exposure for 10 min. of dishes of sterile skim milk; (3) exposure of inoculated agar plates. The second method proved the most convenient and it was adopted as a standard. By means of this method it proved possible to plot out in any given factory the extent of the air-borne infection and to a limited extent to obtain indications of the power of the infection. The extent of air-borne phage infection in New Zealand cheese factories seems remarkable when it is first encountered. One of our early experiences will illustrate this. Two conical Erlenmeyer flasks of 1 l. capacity, plugged with cotton-wool and each containing 600 ml. of skim milk were heated in flowing steam for 45 min. and cooled to 70–75° F. One was retained as a control in the laboratory while the other was taken to a cheese factory a few miles away. While the flask was standing in the cheese-making room the plug was removed from the neck of the flask for 10 sec. and then replaced. The flask was returned to the laboratory and together with the control flask was inoculated (in the usual way with 10 ml.) with the 'single-strain' starter in use at the commercial cheese factory. After incubation, examination of the two cultures showed that the control was quite normal and free from infection, while the exposed flask was 'thin' and the milk contained a high concentration of phage; lysis had occurred overnight. Until this simple

experiment was carried out repeated failures in the factory in question had in years past been ascribed to some abnormality in the milk supply.

By means of exposures of sterile milk in Petri dishes in various places around dairy factories it was found that phage, active against a starter which had been in use for a few weeks, was almost invariably present in the air of the main building of a cheese factory, and that it could spread enough to give positive tests by the dish method to distances of the order of 25 yd. outside the building. As was to be expected, its distribution on any given occasion was conditioned by the weather, in particular wind direction and strength. Wind driving in through an open window could temporarily clear the infection from part of the inside of the building. Similarly, on the lee side of the factory phage could drift for distances of the order of 25 yd. in the open air before it was dissipated sufficiently to make dish tests negative. A true picture of the average conditions around any particular factory could be obtained only by dish exposure tests repeated on several successive days, if possible under varying weather conditions.

(ii) *Creation of air-borne infection by splashing of liquid*

The following experiments were carried out during early attempts to protect the starter from air-borne phage infection. They serve to indicate the mode of formation and the extent of the infection.

I. A skim-milk phage preparation with a titre of 10^{-8} (dilution of 10^{-8} still showed a few plaques when a drop was placed on a plate sown with the susceptible organism) was poured into a shallow tray on a bench in the laboratory. Behind the dish was placed a small electric fan such as is used for ordinary ventilating purposes. An aspirator containing more of the phage preparation was placed so that the glass tap fitted in its tubulure was 8 in. above the liquid in the shallow tray. Dishes of sterilized milk were exposed for 10 min. in the usual way at distances of 2, 8 and 14 ft. from the shallow tray under the following conditions:

(i) With the fan stopped.

(ii) With the fan running (the air passing over the phage preparation in the tray towards the dishes of milk).

(iii) As in (ii) but with phage preparation being dropped at the rate of 80 drops per minute from the glass tap and splashing into the liquid in the tray.

Tests under conditions (i) and (ii) gave negative results throughout, indicating that phage could not pass into the air from the surface of the liquid either with or without an air current. Tests under condition (iii) gave positive results at 2 and 8 ft. but negative results at 14 ft., indicating that the dropping of phage preparation on to the surface of the liquid in the tray produced an air-borne droplet infection which was carried by the air current a distance of between 8 and 14 ft. before it settled out. While the drops were falling it was possible to see a few small droplets rebounding from the surface of the liquid in the tray, but the results indicated that there must have been smaller invisible droplets capable of drifting in the air current. It is easy to visualize how, in a factory, the running of large volumes of whey from taps and the streaming of whey from the rapidly revolving bowl of a separator, create innumerable invisible droplets capable of drifting about in the atmosphere.

II. The same phage preparation as that used in the previous experiment was taken to the Institute experimental factory where a total quantity of 1 gal. was distributed

with a pint dipper over a concrete floor in such a way as to create as much splash as possible. The absence of this particular phage in the atmosphere before the experiment was demonstrated by the dish exposure method. Immediately after the splashing of the whey, dish exposure tests gave positive results all over the room, and a test the next morning still gave a weak positive result (i.e. the exposed milk, after inoculation with the susceptible streptococcus, clotted in 24 hr., but a subculture from this clot into sterile milk showed lysis during the succeeding 24 hr.). The power and persistence of the air-borne infection is well illustrated by this result.

III. Tests for phage in the cheese vat whey and in the atmosphere of the cheese room were made in the Institute experimental factory at the beginning of the season after the whole plant had been overhauled and the walls and ceiling painted. Cheese had not been made for over 3 months. The 'single-strain' starter (HP) was one which had been used regularly for the last 6 years. On the first day of cheese manufacture no phage could be detected in the whey at the time of 'running' when drops of a series of dilutions were placed on a plate culture of the susceptible organism, and a dish exposure test indicated an absence of air-borne phage. Both tests gave frank positives, however, on the second day and thereafter; the titre in the whey at running was 10^{-2}. Dish exposure tests made at intervals through the day gave faint positive or negative results in the early morning, but strong positives soon after the vat taps were opened to draw off the whey. The whey was pumped into an overhead tank in the cheese room and was then allowed to flow to the whey separator (also in the cheese room). Exposure tests made in different parts of the room showed that the whey separator, when running, was a major centre of air-borne phage infection.

In the above instance the starter, both mother culture supplied by the laboratory and bulk culture prepared outside the experimental factory, could be certified as free from phage infection. The cans in which the milk was delivered to the factory never came into contact with whey. Thus the only source of infection was the surface of equipment (which had a more thorough cleaning than would usually obtain in commercial practice) and dust from the environment. Yet after a lapse of 3 months phage appeared in the whey and consequently in the atmosphere on the second day of cheese manufacture.

(iii) *Early attempts to protect starter from air-borne phage*

The tests in which air in cheese factories had been aspirated through cotton-wool had indicated that cotton-wool was capable of trapping air-borne phage quite well. In confirmation of this it was found that mother cultures prepared in conical flasks plugged with cotton-wool could be maintained without failure so long as they were taken well away from the cheese factory for the purpose of inoculation. The milk in the flasks could be sterilized by steam heat and then cooled in the factory building, since the cotton-wool plug prevented phage entering during the cooling process. Needless to say it was essential that the plug should be well formed and fairly firm.

The preparation of bulk starter under existing conditions presented a harder problem because it was impossible to carry it away from the factory for inoculation. But it is not so essential to keep every trace of phage from the bulk starter because it is required only to clot the milk and then to survive in the next propagation until the cheese-making process is finished, unlike the mother culture which is propagated daily from culture to culture for an indefinite period. Therefore it seemed possible that the bulk starter, if

given some protection, might survive even if prepared inside the factory. (At this time the tremendous power of the air-borne infection had not been fully realized.) The first attempt in this direction was made by Mr J. Thompson, manager of the Bell Block Dairy Company, who had a piece of 2 in. piping fitted into the lid of each of his starter cans and used a rubber band stretched round the edge of the lid and can to make an air-tight joint. The can, after the milk had been poured in, could thus be converted into a narrow-mouthed vessel like a flask and the mouth (the 2 in. pipe) could be plugged with cotton-wool. Various modifications of the method of sealing the joint between lid and can were introduced later when the difficulty of making it consistently air-tight with a rubber gasket became apparent. The most successful device up to date is the 'water-seal' lid devised by another manager, Mr J. Ramsay; this is a deep-sided lid which slides loosely over the can and dips into the water in the tub in which the cans of starter milk

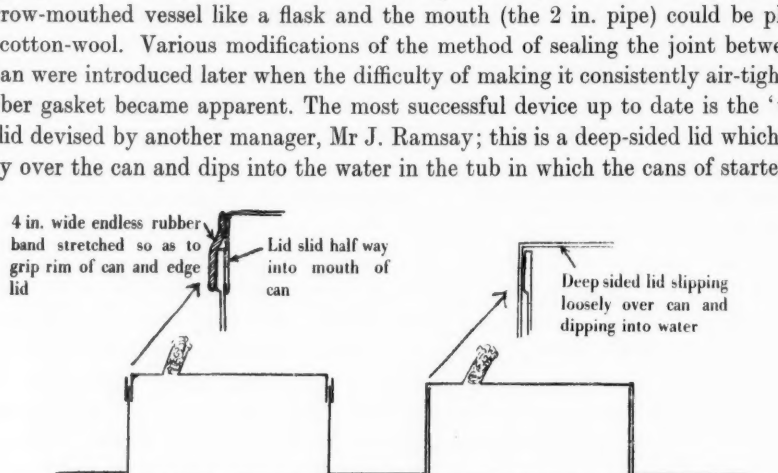
4 in. wide endless rubber band stretched so as to grip rim of can and edge lid
Lid slid half way into mouth of can
Deep sided lid slipping loosely over can and dipping into water

Fig. 2.

are heated, cooled and incubated. Fig. 2 shows starter cans fitted with two of the types of lid which have been used. The difficulty encountered in the provision of a satisfactory seal for the can lid lies in the fact that at least 2 gal. of air space is usually left over the milk in the can. If the can is filled to a higher level, difficulties arise due to expansion of the milk on heating and to a tendency for the water (which must be up to milk level) to boil over the can and later (during the cooling period) to float the can. Most of the air above the milk in the can is driven out during the heating of the milk (up to 200° F. for 1 hr.), and when cooling water is run into the tub there is a sudden inrush of air through the cotton-wool plug. If the plug is made firm enough and deep enough to filter the phage-laden air efficiently, it throttles the flow of air and there is a tendency for leaks to occur through any weak spot in the seal between can and lid. The first (and usually the only) indication of such a leak with any type of rubber gasket is a failure of the bulk starter due to the entry of phage-laden air. With the water-seal lid a negative pressure in the can above a certain value leads to suction of water into the milk which

is evident when the can is subsequently opened and which does little or no harm to the starter *provided the water is phage free.*

For the preparation of bulk starter culture within the factory the cans of milk sealed and plugged with cotton-wool were placed in rectangular tubs of water in which the water-level was slightly above the level of the milk in the cans. The water was raised by means of a steam jet or coil as near to boiling-point as possible (at least 190° F.) and maintained at that temperature for 1 hr. Thereafter, cold water was run through the tub until (by trial and error) water, cans and contained milk reached a steady temperature of between 70 and 75° F. It will be noted that it was not possible to stir the milk in the cans nor to take its temperature direct. This caused no difficulty with cans of 20 or 25 gal. as commonly used in New Zealand. The milk in the cans was inoculated with mother culture by withdrawing the plug momentarily to pour in culture from the mouth of the conical flask. A spirit flame was usually played round the pipe orifice during the inoculation operation.

The adoption of this system of protection against air-borne phage gave excellent results at first and was increasingly widely adopted in dairy factories. After a while, however, there were complaints that although the bulk starter coagulated in the cans and appeared normal when put into the vats in the morning, stoppages in acid production in the vat were relatively frequent. Investigation showed that the initial success had enabled certain 'single-strain starters' to be used consistently for longer periods than before, and that this continued use had gradually raised the concentration of air-borne phage in the factory until it had begun to gain entry to the sealed starter cans in one of the following ways:

(i) Penetration of the cotton-wool plug when air-borne infection exceeded a limiting value.

(ii) Leakage of unfiltered air through an imperfect plug (very common through managers' efforts to economize on cotton-wool).

(iii) Leakage past a rubber gasket while the negative pressure in the can was high.

(iv) Settling into the open tube during inoculation.

It was evident that there was grave danger of phage infection during the cooling period as well as at the time of inoculation.

The general result was that although the protecting devices kept all the phage out on many occasions, there were some instances where enough phage gained entry to cause lysis of the starter in the cheese vat, although it was not enough to cause lysis in the starter can itself. The cases of failure were too frequent to make the system commercially practicable.

(iv) *Attempts at aerial disinfection with sprays*

It was known(8) that a few disinfectants capable of being used in dairy factories (in particular hypochlorite) were very effective in destroying phage. Attempts were therefore made by a spray treatment with hypochlorite solution to reduce or to eliminate the air-borne infection in the room in a factory in which starter was prepared. The possibilities were not exhaustively investigated, and, indeed, the subject of elimination of air-borne infection by sprays is a complicated one as is evidenced by investigations such as those of Baker & Twort(9), but the following conclusions were reached. The only spraying device which can effectively ensure contact between hypochlorite solution and phage is a high-pressure spray gun such as is used in spray painting. Sprays of the type used in

the garden give no appreciable result in reducing infection. Within a dairy factory there is constant reinfection of any room in which a spray has been used. Treatment of the whole factory would be necessary to eliminate the danger of infection of the starter culture, and this would be impracticable more especially since the air-borne infection would need to be eliminated for a period of at least 2 hr. while the starter milk was being cooled and while it was being inoculated. The work of spraying even one room is most unpleasant for the operator, and he would need to wear some form of respirator. The system of spray disinfection is actually being practised in at least two factories with a measure of success, but the idea of its general adoption has been abandoned in the meantime although it may be applied in modified form in the future.

(v) *Destruction of air-borne phage by ultra-violet light*

Sutton (10) showed that ultra-violet light destroys the streptococcal phages and suggested that it might be used to protect cultures from an air-borne droplet infection. We tested the effects of the light given off by 'Westinghouse sterilamps' on air-borne phage in a cheese factory and on phage preparations in the laboratory. It was found, in confirmation of Sutton's results, that ultra-violet light will destroy phage *provided the necessary exposure can be given*, but the practical attainment of this under conditions of constant reinfection is difficult. Trials are still being made to see whether ultra-violet lamps are of practical use for protection of starter cultures. It seems clear already that they may be effective only in conjunction with other safeguards.

(vi) *The isolated starter room*

The experiences detailed above gradually made it evident that continued use of one particular 'single-strain' starter culture in a cheese factory produced such a heavy concentration of air-borne phage in the vicinity of the cheese vats and whey separator, that no system of protection so far devised would safeguard the starter culture to an adequate extent on all occasions. It was decided therefore to provide facilities for preparing the starter at a distance from the factory where the air-borne infection would be either much less or entirely non-existent. An experimental room at Bell Block Dairy Factory was built in 1939. It provided facilities for the preparation of both the bulk starter and the mother culture away from the main factory building and it proved so successful in eliminating contamination of the starter culture with phage that the principles incorporated in it have been adopted as the most promising line of development. About thirty isolated buildings for starter preparation have now been constructed by dairy companies in New Zealand and many other companies have made less elaborate arrangements on the same principle, shutting off a room in the main factory building in such a way that it has no communication with the cheese-making room except through the open air. Figs. 3 and 4 illustrate by diagrams the general arrangement of a typical starter building isolated from the factory.

(vii) *The various safeguards against infection used in conjunction with an isolated starter room*

An isolated starter room has a direct benefit for starter maintenance mainly by virtue of its distance from the factory. The concentration of air-borne phage must necessarily be lower in any place distant from the main sources of generation of the infection.

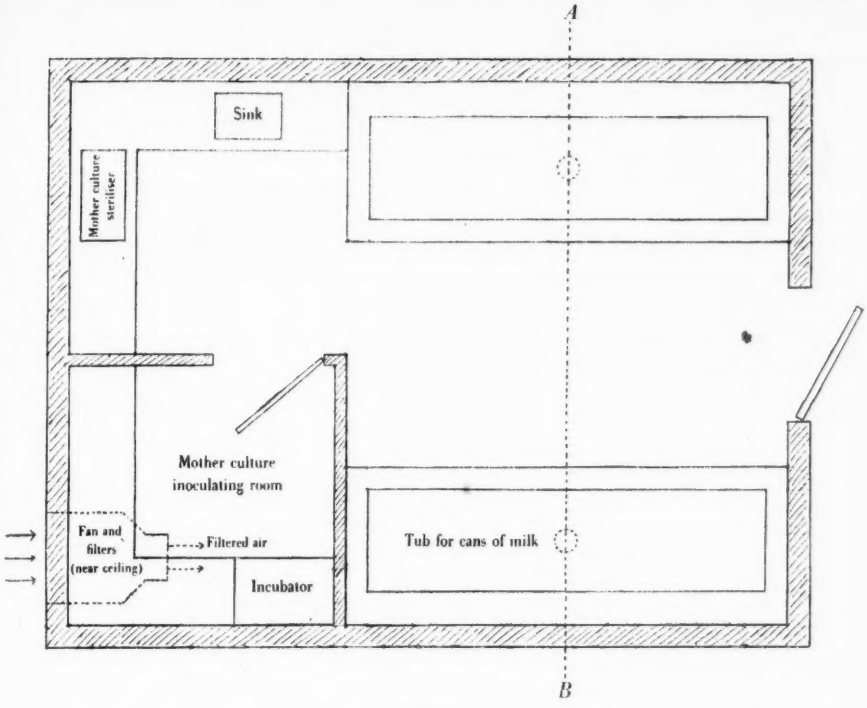
A
Sink
Mother culture steriliser
Mother culture inoculating room
Fan and filters (near ceiling)
Filtered air
Incubator
Tub for cans of milk
B

Fig. 3.

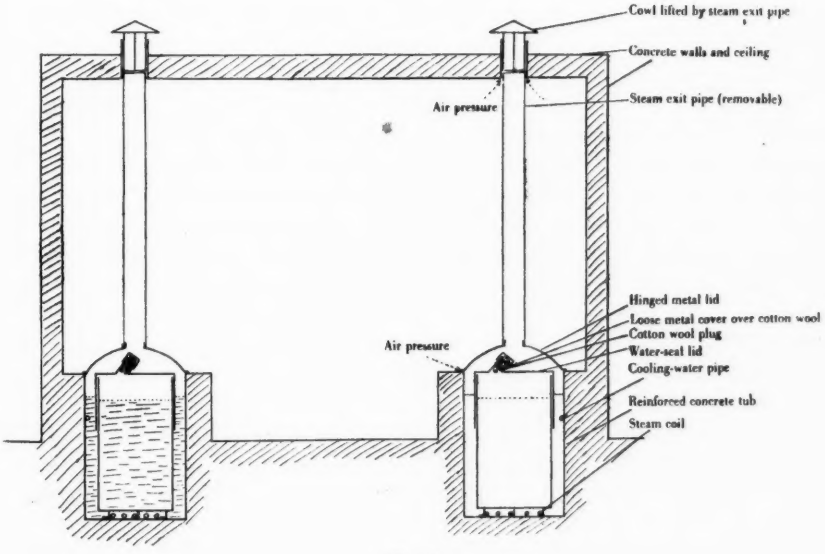
Cowl lifted by steam exit pipe
Concrete walls and ceiling
Air pressure
Steam exit pipe (removable)
Hinged metal lid
Loose metal cover over cotton wool
Cotton wool plug
Air pressure
Water-seal lid
Cooling-water pipe
Reinforced concrete tub
Steam coil

Fig. 4.

Experience in choosing sites for the erection of isolated buildings including tests for the presence of air-borne phage has indicated (as would be expected) that the best site has the following features:

(i) It is as distant from the factory as is consistent with reasonable convenience in supplying steam and water and in transporting milk and starter to and from the room. Distances up to 40 yd. have been used so far.

(ii) It is as remote as possible from the factory drainage system and from the tanks in which whey is stored overnight outside the factory.

(iii) It is in a place where the farmers do not pass when returning home from the factory with milk cans full of whey (whey is almost invariably spilled from the farmers' carts and trucks).

(iv) It is on the windward side of the factory under the most frequently prevailing weather conditions.

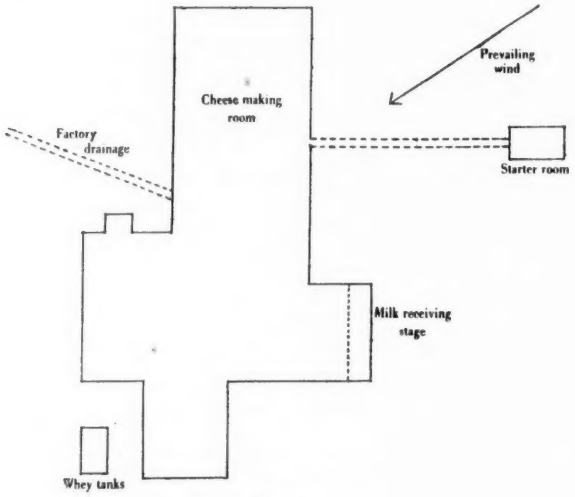

Fig. 5.

Fig. 5 illustrates an ideal lay-out of starter room in relation to factory.

As a result of experience there is an increasing tendency to emphasize distance because it has been found that even in a separate building 40 yd. from the main factory there may be air-borne phage present when a strong wind is blowing from the direction of the factory. The infection under these conditions is, of course, infinitely less than that present in the air of the factory, yet it has, on occasion, given trouble with a starter culture. Occasionally the whey tanks at a factory are not of adequate capacity. The whey froths as it is pumped into them from the separator (it is quite common to have 4 or 5 ft. of foam on top of the liquid whey) and the foam is carried considerable distances by the wind. Similarly, the wind distributes dust from roadways where whey has been spilled. All these factors make it almost impossible to place a starter room where there is no air-borne phage, but at the best only where there is as little air-borne phage as possible.

Thus it has been found advisable not only to have an isolated building but also to have

additional means for trapping any remaining trace of phage which might gain access to the starter. The two main safeguards used in starter rooms to date are:

(i) Sealed vessels with inlets plugged with cotton-wool, viz. conical flasks for mother cultures and cans with 'water-seal' lids for the bulk starter.

(ii) Air filtration. A fan drives air through filters (as used in air-conditioning systems) into the building so that there is a slight positive pressure within. Thus when the outer door of the building is opened infected air does not enter the building. The outlets from the covered tubs, in which the cans of bulk starter milk are heated, are so arranged that the air pressure in the building drives out the steam produced during the heating process. Thus the building is kept dry. The in-driven air from the fan passes through the mother culture room first because dryness is more important there, where the incubator is located. The filters used are not completely effective in trapping air-borne phage, but they remove the greater part. It may be possible in normal times to find more effective filters.

Infection with air-borne phage is so insidious that it has been found well worth while to have these three 'lines of defence' against infection:

(*a*) Distance from the centre of infection, which means a reduced air-borne infection.

(*b*) Filtration of air through special filters.

(*c*) Filtration of air into the cultures through cotton wool.

Even where the complete system is in operation two other measures are used to limit the scope of trouble if phage infection of the starter does occur:

(*a*) At least two different 'single-strain' cultures (i.e. cultures not lysed by the same phages) are prepared separately and mixed in the milk in the cheese vat. If one culture is infected with phage, there is a chance that the other is uninfected, thus enabling the cheese-making process to be carried through with reasonable success.

(*b*) Each can of bulk starter culture (18–22 gal.) is used in two or three vats only, instead of all the cans of starter being mixed and distributed over all the vats. If one can is infected with phage, the trouble is thus limited to a few vats. (It is sometimes not realized by managers that the mixing of an infected can of culture with an uninfected can of the same culture does not give an acid development in the vat midway between normal and zero. Usually the phage in the infected can is sufficient to lyse all the culture resulting in a complete stoppage of acid development.)

An account of all the pitfalls and precautions associated with the use of starters on the lines laid out above may make it appear that the system is too complicated for adoption in commercial practice. This has not proved to be the case in New Zealand. There are now many large cheese factories with up to sixteen vats each of a thousand (Imperial) gallons using starters on this system. There are, of course, many factories still using commercial 'mixed' starters and propagating the culture by dipping it from can to can open to the air in the cheese-making room. But the advantages of 'single-strain' starters (viz. their great activity, their regularity in activity (given adequate protection) and their capacity to make a close-textured cheese) have made many managers persist in attempts to use them. This persistence has resulted in co-operation with the Institute, and this in turn has meant that managers have gained an appreciation of the meaning of aseptic technique. Thus over the course of years each new advance in knowledge on the handling of starters has been introduced more easily into commercial practice until at the present time, in spite of the delicate handling needed by the cultures, large factories make cheese for weeks and even months at a time almost 'by the clock'.

It would be unreasonable to expect a complete freedom from trouble with acid production in commercial cheese factories, but it does appear as if, given complete protection of starter cultures from all forms of infection, there need be no drastic stoppages in acid production due to this particular cause. It is probably more difficult to eliminate regularly all infection from factory and farm equipment, but the nearer this ideal is approached the more rarely will failures in acid development be experienced.

Summary

1. Previous work on bacteriophage infection of starters as a cause of slow acid development in cheese manufacture is reviewed. Air-borne phage seems to be the form of infection which is most insidious and most difficult to exclude. Hypotheses are developed to explain the sequence of events observed in commercial practice. The spread of phages as dust or droplet infections seems to explain the gradual increase of trouble with a given starter culture within a single factory and also throughout an entire dairying district.

2. When phage is established in the surroundings of a dairy factory a cycle of infection can develop. Besides the air-borne droplet infection there may be daily reinfection of cheese milk from incompletely sterilized factory and farm equipment which comes into contact with the milk. A description is given of methods which have been used to determine in any given case whether slow acid development is due to phage infection of the starter itself or to infection of the milk from factory or farm utensils.

3. The problem of preventing phage infection of the starter culture is fully discussed. The extent of the air-borne infection to be guarded against is indicated. The equipment which has so far been developed in New Zealand for the aseptic handling of starter cultures is described in detail. The most promising lines of development are indicated.

The authors' thanks are due to Prof. W. Riddet and Dr F. H. McDowall for their constant interest and advice.

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323. OBSERVATIONS ON CHEESE STARTERS WITH REFERENCE TO BACTERIOPHAGE AND THE PHAGE-ORGANISM RELATIONSHIPS OF STRAINS ISOLATED

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In recent years 'slow-working' cheese and starter failure have caused considerable difficulty in the manufacture of hard-pressed cheese, and the losses due to such 'failure' have become increasingly important. Since continental sources of commercial starters have been denied to cheese-makers in this country, it was felt that a survey of the starter position might yield useful information.

During the 1942 cheese-making season starter samples from cheese factories and other sources, including dairy schools, were examined at frequent intervals for susceptibility to bacteriophage from the whey. During the same season visits were made to factories and many dairy schools in order to establish the ultimate sources of starter in use in this country.

I. General investigation of starters

Methods

The starters were tested for susceptibility to bacteriophage obtained from the corresponding wheys, as follows:

Sterile sampling outfits were supplied. The laboratory staff at the factory was instructed to use the standardized dropping pipettes supplied to them and to inoculate each of two 25 ml. McCartney bottles of sterile milk. One bottle was inoculated with 1% of mother starter and the other with 1% of the bulk starter in use. A sample of whey from the corresponding cheese was despatched and the samples, already coagulated and ready for testing, usually arrived on the following morning. If a delay in the post occurred the starters, on arrival, were inoculated into tubes of sterile separated milk and tested the following day.

The starter test for the susceptibility to bacteriophage used is a modification of the activity test (1, 2, 3) and was carried out as follows: Approximately 2·5 ml. of an 18 hr. old starter culture which had been incubated at 22° C. were added to 7·5 ml. of sterile milk in a test tube ($6 \times \frac{3}{4}$ in.) and thoroughly mixed by pipetting. 1 ml. of this 25% starter dilution was added to each of 2 vol. of 25 ml. of sterile milk in McCartney bottles. One bottle in each pair acted as a control, while to each of the others was added 2 drops of the corresponding whey which had previously been filtered through a Seitz filter. After incubation for 6 hr. at 30° C., 10 ml. portions were pipetted into a basin, the pipette was rinsed with 10 ml. of water which was added to the contents of the basin and the whole titrated with $N/10$ NaOH. When the addition of whey resulted in at least a 10% depression of the titration figure, bacteriophage was presumed to be the cause. The presence of phage was confirmed by the isolation of plaques from yeast dextrose agar (Y.D.A.) plates. The relationship of the various phages and strains thus isolated is discussed later.

In the activity test adopted by Meanwell (1, 2) for the control of starters at cheese-making factories the use of a laboratory original mother starter against which the factory submother and bulk starters are tested, is of great importance. In the present investigation original mother starters which could be kept free from phage contamination or away from the factory were not available, but many of them were retained here and were

used in work during the 1943 season with success. The estimate of the incidence of phage reported, therefore, is probably lower than it should be, since before arrival or during ransit certain strains in the starters may have phaged and without the original mother starter there was no standard against which to assess them.

It should be noted throughout the work reported in this paper that all acidity tests, phage isolations and cross-tests have been carried out at 30° C. It is possible therefore that some phage races which are more active at 37° C. than at 30° C. or which react only at the higher temperature may have been missed (2). Hunter (4), working with several strains of *Str. cremoris* and their appropriate phages, has reported, however, that all of the starter phages reacting at 37° C. were active also at 30° C. Meanwell (5) has also shown that some phages of starter strains are active at 37° C. and not at 30° C.

Ten factories distributed throughout England and Wales, and representative of the main cheese-making districts, contributed 131 sets of samples for examination, and practically all of them (121) consisted of samples of both mother and bulk starter. Of the twenty miscellaneous samples received sixteen were from dairy schools and fourteen of these samples were obtained from one school, K. As will be seen from Table 1, thirty-three of the 151 total samples tested appeared to show evidence of phage, and from thirty of these starters phage-susceptible strains of lactic streptococci and their corresponding phages have been isolated.

With the exception of factories E and F, samples were not received regularly, since the tendency was to send samples only when starter 'slowness' occurred. Thus the proportion of phaging samples in the table has been unduly increased, and this disproportionate increase is also true for the dairy school K where the six phages isolated in May and the two obtained in June probably represented the only eight of the season, since 'slowness' there was rare or absent during July and August. Similarly, at factory H the four phaging samples were due to starters which had also phaged elsewhere, and when the starter was again obtained from another source it remained comparatively free from phage for the rest of the season. On the other hand, the absence from the table of the non-phaging starters from school K is balanced by the absence of an equally numerous collection of phaging starters from factory A where it is known that starters phaged regularly and frequently throughout the season.

In view of these irregularities of sampling it is difficult to analyse statistically the distribution of phage occurrence during the cheese-making season.

Findings and discussion

On the basis of these rather limited figures, supplemented by information received at the factories, the following points are of interest:

(1) It would appear that phage is prevalent not only in May but also in August, and these figures support the widely held belief that starter 'slowness' is particularly prevalent in May. Regarding the incidence of phage in April and September, the paucity of samples examined—four only—precludes even tentative conclusions. Large-scale cheese-making is, of course, usually limited during both these months.

(2) It will be seen from Table 1 that phaging is geographically widespread in this country. It may be noted in this connexion that the same starter B, from which eight strains were isolated, phaged at widely separated places on 3 May at factory B (Somerset), on 4 May both at dairy school K (south England) and at factory H (Shropshire), and

Table 1. *Seasonal distribution of starter samples showing presence of bacteriophage in the whey*

	Source of samples																							
	Factories																		Miscellaneous Berkshire Lancashire Somerset S.W. Scotland S. Wales Warwickshire					
	A Shropshire		B Somerset		C Cheshire		D Shropshire		E Dorset		F Cumberland		G Cheshire		H Shropshire		I Dorset		J S.W. Wales					
Treatment of milk...	Raw or pasteurized		Pasteurized		Raw or pasteurized		Raw		Pasteurized		Raw		Raw		Raw		Pasteurized		Pasteurized		Mainly raw		Totals		% phaging samples
	P	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T	
April	.	3	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	4	.
May	4	9	2	2	2	3	1	2	.	5	.	7	.	3	3	5	.	1	.	.	6	12	18	49	36·7
June	1	1	.	2	.	1	2	4	.	5	.	9	.	5	1	2	.	5	.	1	2	4	6	39	15·0
July	.	2	1	2	1	3	.	1	.	4	.	7	.	3	.	5	.	3	.	2	.	3	2	35	5·7
August	.	3	2	2	1	1	.	2	.	3	.	4	.	.	.	.	.	.	2	5	.	.	5	20	25·0
September	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	1	1	2	4	.
Totals	6	19	5	8	4	8	3	9	.	18	.	27	.	11	4	12	.	9	2	10	9	20	33	151	

P = phaging samples; T = total samples tested.

again when reintroduced into factory B on 8 May. Yet it did not phage at factory E when used on 4 and 6 May. A totally different starter, Y, phaged at dairy school K on 16 May, on the 18th and 19th at factory A (Shropshire), and on the 25th at factory H.

(3) Two factories E and F were free from phage in the whey and 'slowness' in the cheese-making throughout the season. Samples of their starters, originally obtained from different sources, were tested at least once per week. (Again during the 1943 season when forty-four samples were tested from factory E and thirty-two from factory F the same freedom from phage was evident.) Factory G was also free from phage on all occasions when the starters were tested. In none of the last three factories were any special precautions adopted to keep the starter protected from whey contamination, while at E the mother starter was kept in the cheese-making room protected only by muslin.

(4) Some cheese-makers have held the view that starter 'failure' has been more prevalent since the introduction of pasteurized milk. The number of phaging samples obtained from sources making cheese from raw and from heat-treated milk was calculated, and it was found that the percentage of phaging samples was practically identical for both raw milk and pasteurized milk cheese (23%). Further field work during 1943 has shown that the same starters will repeatedly phage at both 'raw milk' and 'pasteurized milk' cheese factories, but that 'slowness' may undoubtedly be masked in the early stages with a weak phage or where only a relatively small amount of cheese is manufactured, if raw milk is used.

(5) From information obtained during visits to the cheese-making centres it has become evident that the original source of most of the starters now in use in this country has been commercial powder starters. Consequently many of the starters distributed by dairy schools are probably from the same original source and may indeed consist of similar or identical strains. This is also often true of factory starters, especially when one factory supplies a neighbouring factory with starter. Dairy schools also exchange starters.

(6) Cheese-makers often prefer starter from a particular source, but it is probably true to say that there is little to choose between them, as no supplier in this country appears to make up starters from strains newly isolated.

II. Phage-organism relationships of the strains isolated

Early in this work it was realized that interchange of starters was common between dairies, especially when 'slowness' occurred, when a starter had urgently and quickly to be replaced. The difficulty of obtaining reliable information regarding the origin of these commercial starters was considerable, and it has been shown that a creamery, after an outbreak of 'slowness', having obtained what it considered to be a new starter from a different source, had in fact reintroduced the same susceptible starter masquerading under a new designation.

It was hoped that a study of the phage-organism relationships of the various strains of organisms and phages isolated would clarify the interrelationship and possible identity of many of these commercial strains, and that it might be possible to use the results to avoid the use of certain strains immediately after a particular bacteriophage had made its appearance in a creamery.

General methods of isolation and purification of strains and phages

Each susceptible starter was plated on Y.D.A. and 12–24 colonies were picked into yeast dextrose broth (Y.D.B.) and tested against the filtered whey. The phage-susceptible strains so obtained were purified by three successive platings on Y.D.A. After each stage of strain purification two well-isolated colonies were picked into Y.D.B. and after growth were tested against the filtered whey to make sure that the susceptible phaging strain had been isolated in each instance.

The corresponding bacteriophage race was isolated and purified by three successive pickings of plaques from Y.D.A. plates using the previously purified strain as host. Plaques were picked into Y.D.B. and, after clearing, the broth was passed through a Seitz filter and stored in a refrigerator at 4–6°C. The same bulk phage preparations were used throughout this investigation.

Although this method of isolation worked well with many of the starters, several starters showed a considerable reduction in the milk activity test and yet failed to yield phage-susceptible strains when colonies from each starter were tested in broth against the whey. Consequently the broth cultures were inoculated into separated milk, incubated overnight at 22°C. and the following day a 1% inoculum of a freshly coagulated culture tested by the activity method against the whey. In every instance it was possible to recover one or more phaging strains, and hence for some strains the milk activity test appeared to be more sensitive for phage isolation than the observation of clearing in broth. Table 2 summarizes these results.

Table 2. *Recovery of phaging strains by milk activity and broth tests*

Starter no.	Total number of strains tested for phage susceptibility by milk and broth tests. All non-phaging by broth test	Number of strains phage-susceptible by milk activity test only
161	11	10
166	12	6
174	16	3
185	12	8
209	12	1
216	12	9
219	12	2
223	12	9
262	11	8

It is possible that the bacteriophage concentration of these wheys was low and had been further weakened during the interval between production and examination of the strains. In broth the bacterial multiplication was probably greater than the lysis. Thus, since the broth test depends on clearing, if the phage be weak, the equilibrium will be shifted in favour of bacterial growth, and an apparent 'non-phaging' reaction will be obtained. In milk, however, the test depends on a quantitative difference of acidity between the control and the effect of added whey, and even a dilute phage is able to assert its effect. It was observed that the strains from these starters were often either low or very moderate acid producers in sterile separated milk, and required incubation of 12 or more hours at 30°C. to produce sufficient acidity. In Y.D.B., however, there was the stimulation due to the yeast extract, and turbidity could be observed after the usual interval of 4–5 hr. The additional incubation period in milk would allow a weak phage, outpaced in broth by vigorous bacterial growth, to increase and reveal itself.

After removing 10 ml. of milk for the 'activity test' titration, the remainder was acidified with sterile $N/10$ lactic acid until coagulation occurred, centrifuged for 5 min. at 2000 r.p.m. and the supernatant liquid passed through a Seitz filter. This filtrate replaced the factory whey for the further purification of the bacteriophage.

Experience with additional starter phages during the 1943 season has shown that in spite of the apparent failure of some picked plaques to show clearing in broth, phage could nevertheless be demonstrated by filtering the overgrown culture. Clearing or plaques could then be obtained with the filtrate. Unfortunately, none of the overgrown broth cultures was filtered during the 1942 season to ascertain whether phage was present.

Some of the phages presented difficulties in purification. Thus the first, second and third series of plaques of the five phages 170/1/5/7 and 185/1/4 were only obtained after several repetitions. Further, when these plaques were inoculated into Y.D.B. containing dilutions of the host culture, clearing was not obtained and the plaques had to be re-inoculated several times in order to obtain evidence of bacteriophage. Both Whitehead & Hunter (6), working with starter phages, and Burnet (7), working with phage of staphylococci, observed that some phages, although producing plaques on agar, fail to show clearing in broth.

Because of its simplicity, the broth test was used to investigate the phage-organism relationships. It was not fully realized at the time that a milk-activity test, as was shown earlier in this paper, may be more sensitive for the isolation of some phages and that milk might therefore be a superior medium for the cross-phaging tests. Indications of this were obtained later in the work on nascent phage.

Several strains were usually selected for study from each starter, to allow for any difference between strains. Altogether sixty-four strains with seventy-two of their corresponding phages were used in this work.

Experimental method of investigating the phage-organism relationships of the strains isolated

The reactions of the various phage races and strains were studied by using a modification of the broth test as follows:

1 ml. of an 18 hr. old culture of the strain grown in Y.D.B. at 30° C. (generally showing heavy growth) was diluted in 9 ml. of ¼-strength Ringer's solution and 2 drops (approximately $\frac{1}{10}$ ml.) added to 1 ml. of Y.D.B. contained in small sterile glass tubes ($3 \times \frac{1}{10}$ in.). 1 drop of a Y.D.B. filtrate of the bacteriophage race under test was added. Control tubes contained diluted culture only, and uninoculated controls aided in the final reading of turbidity. All tubes were prewarmed at 37° C. for 1 hr. and were incubated at 30° C. for at least 5 hr. The tubes were shaken and readings taken at 1 hr. intervals. Absence of turbidity (clearing) in the tube containing the bacteriophage was recorded as positive (+). All tubes were left overnight on the bench at room temperature, a final reading being taken in the morning.

The results thus obtained are recorded in Table 3.

Results and discussion

A consideration of the phage-organism reactions contained in Table 3 brings out the following points:

(1) (*a*) Strain 1P5 was isolated during the 1941 season from a starter originating from a Somerset factory and reacts with the phages of the Y strains. Strains Y/1/4/6/15/16 were isolated from starter Y in use in the same factory during the 1942 season. The latter starter is seen to give rise to two distinct types of strains, Y/1/4/6 and Y/15/16.

It will be noticed that none of the Y strains or strain 1P5, or their phages, show any other reactions with any other strains or phages. Since this factory is known to have

carried this active starter for many years, and as the strains and phages of Y and 1 P 5 are so specific in their reactions, it is highly probable that starters Y and 1 P 5 were identical. The nascent phage reactions discussed later in this paper seem to confirm this contention.

(*b*) Strains B/7/25/27 show very close relationship. On the other hand, strains B/3/4, although isolated from the same mother starter, do not react with any other strain from starter B. Thus a single starter is again seen to give rise to more than one type of strain, and it is possible that in spite of prolonged daily subculture—when one might expect a commercial starter to resolve itself into a single strain—starter B contained several types of strains. Strains 156/1/2/5, isolated from another sample of starter B while in use at school K, and strain 158/2, isolated from starter B while in use at factory H, are very similar and closely resemble the reactions of strains B/7/25/27. 144 F was isolated as the active strain from starter in use at school K in October 1941 and was obtained by the school from the same source of supply as starter B. The strain is practically identical in its reactions with strains B/7/25/27 and 158/2.

(*c*) Strain 167/11, which was isolated from starter B while in use at factory B, does not react with any of the phages of the other strains of starter B, although most of them react with phage 167/11. Thus starter B probably contained a third type of strain. A further indication that this strain is related to the other is provided by the nascent phage reactions.

(*d*) Strain 172/3 shows some reactions with the phages attacking the strains of starter B, while phage race 172/3 reacts with strains B/27 and 144 F.

Our records ascribe it to a northern source, and it is quite likely that, like starter B, strain 172/3 originated in starter powder from the same source known to supply both places.

(*e*) Strain 174/11 reacting with phage races *b*/3/4 was isolated from a commercial starter and little is known of its origin.

(2) (*a*) Strains 161/3/8 were isolated from a starter in use in factory C. Little is known of their origin except that they came from a reputable commercial source. In the laboratory they were found to react with phages 198 and 205 originating from wheys in use at factory A and dairy school B, with neither of which, as far as could be ascertained, were they related. None of the other strains react with the phage races of 161, which bears out this complete lack of relationship.

(*b*) Strain 198/1, isolated from starter Y while in use at factory A, fails to react with any of the phage races of *y* or 1 *p* 5, and none of its reactions with the other phage races suggests its relationship to these two starters. No explanation can be offered for this complete lack of reactions, except that at factory A at this time several starters were in use, and were changed almost daily owing to phage trouble. The starter sent to us as Y might have been a resistant form, or might have consisted of the residual flora after phaging.

(3) Strains 166/7/8 came from a northern starter in use at factory F from which no phage was recovered throughout the season. As starter 223 it phaged at factory A giving rise to strains and phages 223/13/18. Strain 166/8 reacts with several of the 278 phage races with which no relationship could be traced. Phage 166/7 reacts, however, with strains 216/9/12 originating in another starter from the same source.

(4) Strains 170/1/5/7 were isolated from starter 170 which originated from the same

Table 3. *Phage-organism reactions recorded in broth*

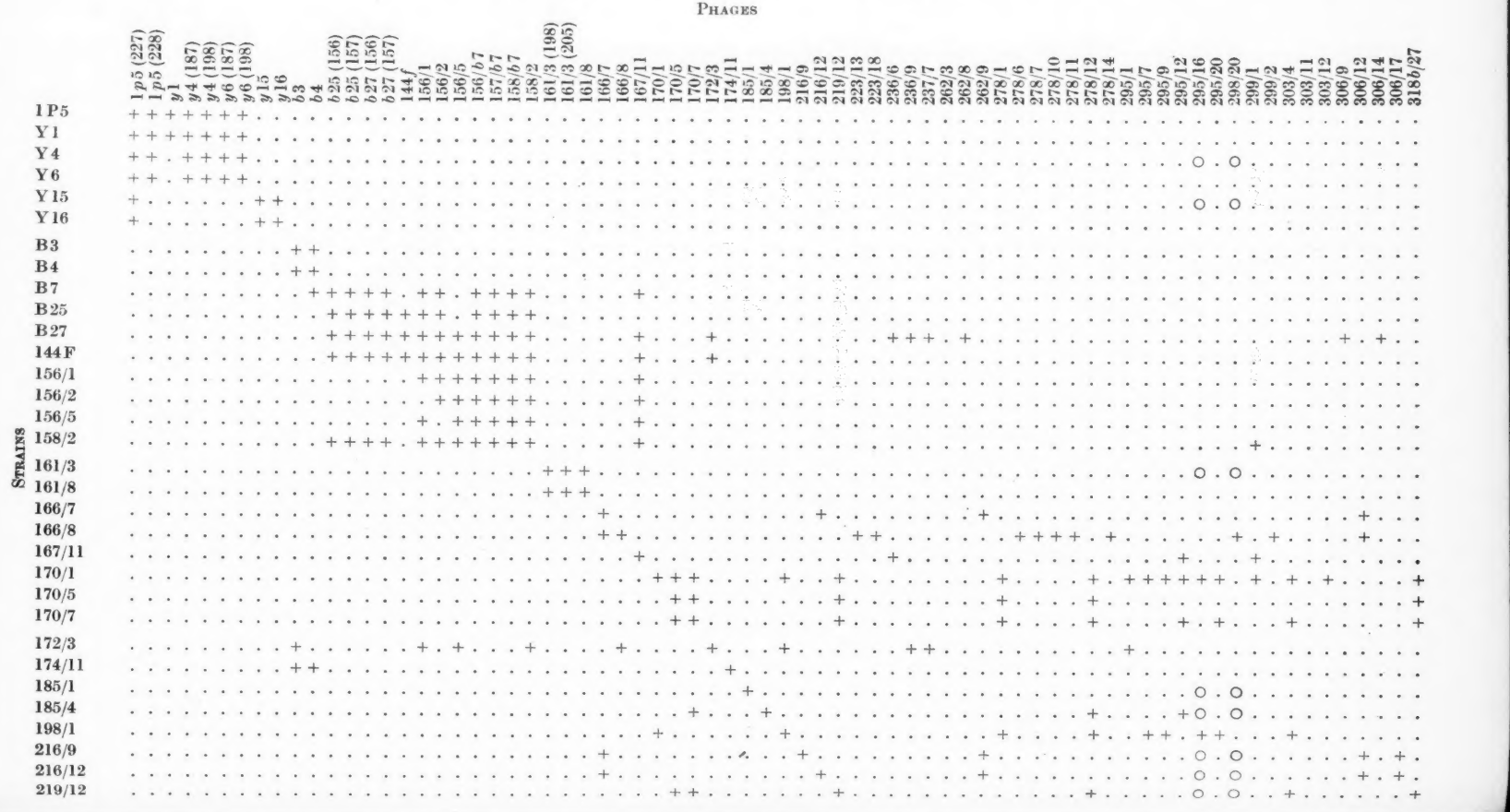
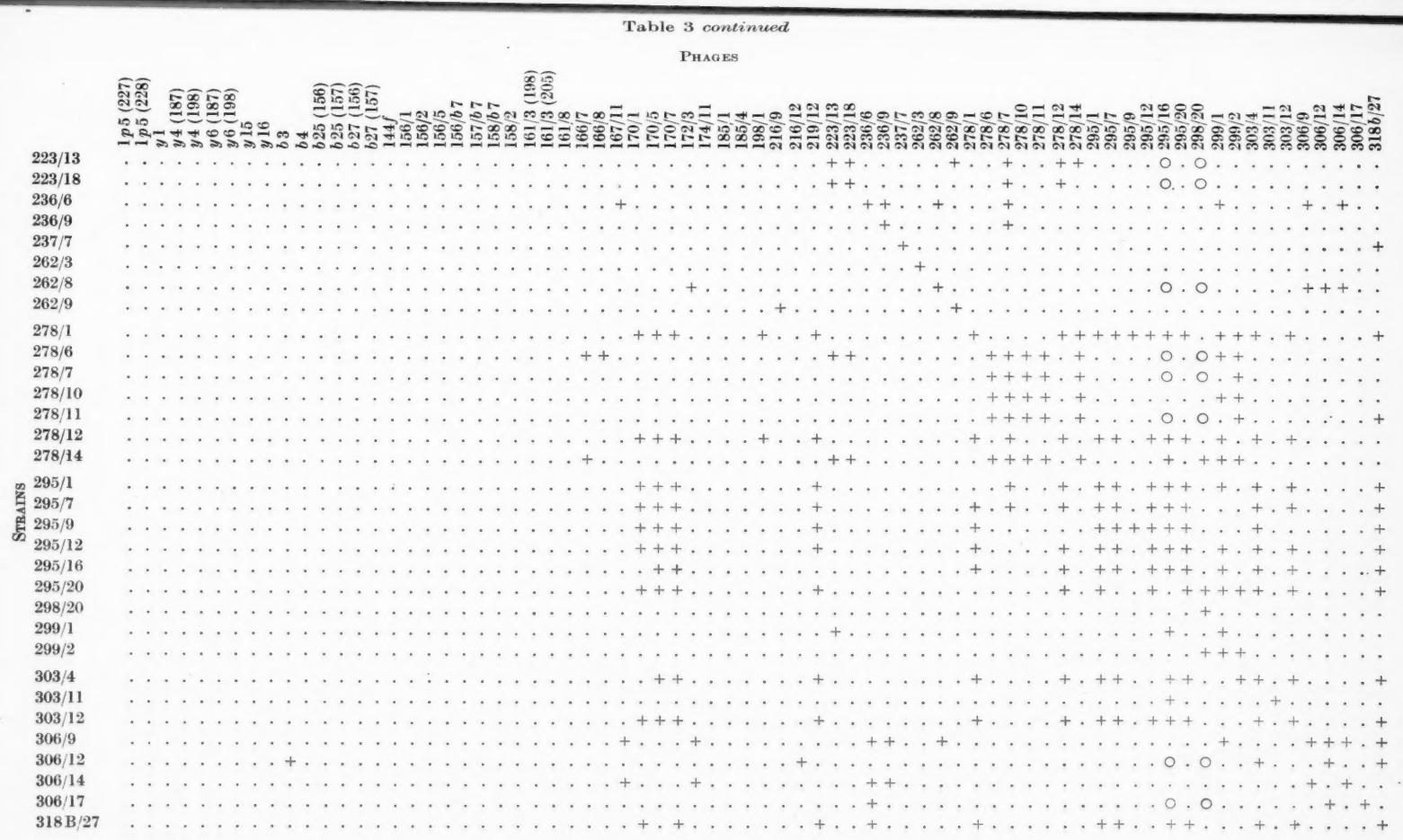


Table 3 *continued*

PHAGES

Table 3 *continued*



+ = phaging. ○ = no test. . = no reaction.

Somerset factory as starter Y with which, as far as it was known, it was not related, nor do the strains react with the strains or phages of Y or 1P5. Strain 219/12 was isolated from starter 170 when in use at factory H. The strain reacts with phage races 170/5/7, and conversely strains 170/1/5/7 all react with phage 219/12. Thus their identity is not in doubt.

Table 4. *Reduction in acidity due to nascent phage. Titratable acidity in ml. N*/10 NaOH *per* 10 *ml. milk*

	Nascent phage 1*p*5			Nascent phage *y*/1		
Culture	1% test culture	1% test culture + phage 1*p*5	1% test culture + phage 1*p*5 + 1% 1P5 culture	1% test culture	1% test culture + phage *y*/1	1% test culture + phage *y*/1 + 1% Y/1 culture
1P5	5·1	2·7	.	4·2	2·4	2·8
Y/1	4·8	2·6	2·8	3·8	2·4	.
B/27	5·8	6·0	5·9	4·9	5·6	5·0
144F	5·2	5·4	5·2	4·3	4·9	4·6
156/5	5·7	6·1	5·7	4·9	4·8	4·8
167/11	6·0	6·4	5·8	4·7	4·7	4·7
170/1	4·8	4·7	4·7	3·8	4·0	3·7
198/1	5·5	6·1	5·6	4·0	4·2	4·1
278/1	5·2	5·3	5·1	3·9	4·5	4·2
295/20	4·4	4·4	4·6	3·7	3·8	3·8
298/20	4·4	4·6	4·5	3·6	3·5	3·5
299/2	*4·5	*4·3	*3·0	*4·0	*4·4	*2·8
303/4	4·7	4·6	4·5	4·5	4·3	4·4
318B/27	4·6	4·8	5·1	4·3	4·1	4·5

	Nascent phage 298/20			Nascent phage 299/2		
Culture	1% test culture	1% test culture + phage 298/20	1% test culture + phage 298/20 + 1% 298/20 culture	1% test culture	1% test culture + phage 299/2	1% test culture + phage 299/2 + 1% 299/2 culture
1P5	*4·1	*4·0	*2·9	6·2	6·3	6·4
Y/1	*4·0	*4·1	*3·1	6·6	6·6	6·7
B/27	*5·7	*5·4	*3·8	7·0	6·2	6·1
144F	*5·5	*5·3	*3·4	6·7	6·5	6·7
156/5	*5·2	*5·2	*4·1	6·3	6·4	6·5
167/11	*5·7	*5·5	*3·1	6·0	5·7	6·3
170/1	4·1	4·2	4·2	5·6	5·2	5·7
198/1	5·0	5·0	5·1	5·7	6·0	5·7
278/1	5·0	5·0	5·0	6·0	5·4	5·6
295/20	4·8	4·6	4·6	5·4	4·7	4·8
298/20	4·0	2·4	.	*6·6	*6·6	*4·1
299/2	5·0	2·9	3·2	3·9	2·8	.
303/4	5·8	5·6	5·7	6·0	5·7	5·7
318B/27	4·8	4·7	4·8	6·6	6·1	6·4

* Series of titrations showing nascent phenomenon.

The strains of 170 and 219/12 show reactions with phages 278/12, 303/4 and 318*b*/27. Conversely, strains 278/12, 303/4 and 318B/27 react with the phages of strains 170 and 219/12. Thus a common origin or similarity in 'make-up' of these strains is indicated. Starters 303 and 318 came from the same source, but not starter 278. Strains 170/1/7 react with phage races 295/12/20, while all the six strains from starter 295 show consistent reactions with all the phages of 170. Starter 295 came from the same northern distributing centre, and the relation therefore between starters 170 and 219 on the one hand and 303, 295 and 318 on the other is unlikely to be merely accidental.

(5) Strains 174/1 and 185/1/4 were from two distinct starters derived from the same commercial source, and their reactions show them to be unrelated to most of the other strains.

(6) Starters 236, 262 and 306 were known to come from the same source. The reactions of all their strains and phages do not, however, point to any common origin. Further, many of these strains and phages react with many of the other strains or phages, a tendency which is shown in many instances.

(7) The strains of starter 278 were derived from a commercial mixed starter supplied by school K. They all react with phage 299/2, the strain of the latter originating from the same source as starter 278.

(8) Strain 318B/27 was isolated from a starter from the northern source and shows reactions with phages of strains 170/1/7, 219/12, 295/1/7/16/20, 304/4/12, all attacking strains derived from the same source. Its reactions with phage races 236/6 and 278/1 were not anticipated.

(9) Summarizing the results of Table 3 it will be seen that the phage tests help to unravel the relationship between many commercial starters and confirm the identity of some.

Not all the strains isolated from the same starter give identical reactions against any one phage. Thus the cross-reactions of any one starter cannot be judged by that of any one of its strains, and the greater the number of strains examined the more representative those reactions will be for any particular starter. In short, commercial starters have been found to be composed of strains or groups of strains showing distinct reactions. There is some cross-reaction between the strains of a number of starters circulating in commerce.

III. NASCENT PHAGE REACTIONS

Evans(8) has described the nascent phage phenomenon in connexion with streptococcal bacteriophage, and more recently both Anderson & Meanwell(2) and Hunter(4) have referred to the importance of this reaction in the successful management of starters. Thus, although a certain phage race will not attack a certain streptococcal strain it may affect that organism in the presence of its own homologous strain.

In cheese-making there are several reasons why such a reaction may take place. When a starter has phaged in the factory it is still possible for many of the organisms to survive in inaccessible parts of the dairy or, as observed with some starter strains, the phaging may not be complete. Thus, the substitution of a strain which normally does not react with the particular phage race present in the dairy gives no guarantee that the newly chosen strain will not be affected by nascent phage. If these new strains are being introduced into a factory for the first time and the phages for these strains have not yet been obtained, there is another dangerous possibility: when two or more of these strains are cultivated separately at the dairy and mixed in the cheese vat, if one of the strains has been lysed, its phage, even though inactive normally against the other strains, may affect them when in the nascent state.

In order to obtain data on the frequency of this phenomenon amongst the strains and phage races isolated, a representative selection was made of vigorous strains suitable for cheese-making. The following fourteen strains and their corresponding phage races were tested: 1P5, Y/1, B/27, 144F, 156/5, 167/11, 170/1, 198/1, 278/1, 295/20, 298/20, 299/2, 303/4, 318B/27.

Method

The milk-activity test was used for this work and was carried out as described in Part I of this paper. Three bottles of milk were inoculated with a 1% inoculum of each strain and one bottle was retained as a control. Of the inoculum 1% was used, since this would frequently be the *maximum* concentration of each strain as it might occur in starter. Two drops ($\frac{1}{10}$ ml.) of the filtered phage under tests in Y.D.B., were added to each of the other two bottles and a 1% inoculum of the homologous strain of this phage race was added to one of the bottles containing phage. Two drops of sterile Y.D.B. were also added to the control milk to compensate for the stimulatory effect of the yeast dextrose medium in the milk cultures containing the phage filtrate.

Fourteen strains together with their phages have been investigated. When the nascent phage phenomenon was evident, the acidity of the control milk was considerably greater than that of the milk which received inocula of both strains and also of phage. The milk containing an inoculum of phage and of one strain only, acted as a control and revealed any phage races which cross-phaged with the original test organism.

Results and discussion

(1) Only four phages showed the nascent phenomenon. The titratable acidity figures of the four sets of experiments yielding a positive result are given in full, since they also show the lack of reaction of the strains (Table 4).

(2) (*a*) The evidence for the probable identity of strains and phages 1P5 and Y/1 has been discussed earlier. It will be seen that strain 299/2 which is attacked by nascent phage 1*p*5, is also attacked by nascent phage *y*/1. Similarly nascent phage 298/20 attacks both of these strains equally.

(*b*) The close relationship of strains B/27, 144F and 156/5 has already been discussed.

(*c*) Strain 167/11 was found to be distinctly different in its reactions from the other strains also isolated from starter B, but in its sensitivity to nascent phage 298/20 it is similar to the other strains of starter B. Thus it is possible that the nascent phage reaction points to a fundamental relationship between strains and could be used as a further means of classifying related strains and phages.

(*d*) Strain 298/20 is not affected by the nascent phage 299/2, whereas strain 299/2 is lysed by phage 298/20. Here, too, the nascent phage reaction points to a close relationship of these two strains.

Summary

1. Frequent examination of the mother and bulk starters from several cheese-making creameries during the 1942 season has shown bacteriophage to be prevalent in some factories and apparently absent from others. Outbreaks of phage attack could not be correlated with heat treatment of the cheese milk, and were geographically widespread over the country.

2. The phage-organism relationships of these starter strains and their phage races were studied, and on this basis it was possible to arrive at the relationship and in some cases the identity of some of the strains circulating in commerce. It was found that for the isolation of some phaging strains, milk was a more sensitive medium than broth. Commercial starters appear to be composed of more than one distinct strain, and no starter can be judged by the reactions of one of its component strains.

3. The nascent phage phenomenon was found not to be common in the selected starter

strains. The reaction itself may, however, possibly be used to demonstrate fundamental relationships between strains.

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324. THE HEAT RESISTANCE OF THE BACTERIOPHAGES OF CHEESE STARTER WITH OBSERVATIONS ON THE ESTIMATION OF PHAGE CONCENTRATION

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In the control of slow acid development at cheese-making factories due to attack on the starter by bacteriophage, it is of some practical importance to define the heat treatment necessary to destroy phage. Several workers have reported on the heat resistance of phages which attack lactic acid streptococci. Mazé(1) showed that phage would survive heating in a test-tube of sterile milk for 5 min. at 65° C. and at 75° C. but not at 80° C., but if the host culture was also added to the medium and the tubes were incubated for 2 or 4 hr. at 20–22° C. before heating, the phage survived only at 65° C. He concluded that in sterilized milk free phage is more resistant to heat than phage which had penetrated the bodies of the organisms, although he suggested that the acidity of the 'microbic bodies' might be responsible for the difference. Whitehead & Hunter(2), working with nine phage races, reported that 'their thermal death-points are in the region of 70–75° C. (half-hour exposures at *p*H 6)'. Nelson, Harriman & Hammer(3) found that filtrates of 'inhibitory principle' (later demonstrated to be bacteriophage) derived from slow butter

cultures, resisted, at approximately neutral reactions, heating at 70° C. for 10 but not for 15 min. These workers showed that at lower *p*H values the 'inhibitory principle' was less resistant to heat, but it appeared to be more resistant than the corresponding organism.

Evans (4) has also investigated the heat resistance of streptococcus phages of four serological types and found that her phage races A and B were inactivated by heating for 1 hr. at 60° C., phage race C at 65° C. and phage race D at 63° C. Evans & Sockrider (5) found that at a titre of 10^{-6} phage race E survived 74–75° C. They state, 'the temperature of inactivation was thus shown to be higher by 10 degrees for phage E than for any other known streptococcic phage'.

Yakovlev (6) showed that phage isolated from starter used in the preparation of margarine had a high heat resistance. Thus in small volumes of a medium containing serum it resisted 80° C. for 10 min. In large volumes of whey it survived heating at 90° C. for 5 min. In the dried condition the phage resisted heating in an oven at 95° C. for 2 hr.

Experimental

Sources of phage

The host strains and the corresponding phages were obtained during the examination of commercial starters and had been purified (8). The twenty-six phages used for heat-resistance tests were selected from some eleven groups which had been shown from the cross-phage tests previously reported to be unrelated (8). It was hoped, therefore, that any phage of outstanding heat resistance might be discovered.

Estimation of phage concentration

Three methods were used:

(*a*) A milk-activity test depending on acid development;

(*b*) A broth test in which phaging was indicated by clearing of the turbidity;

(*c*) The formation of plaques on solid media.

For these tests dilutions of the phages in $\frac{1}{4}$ strength Ringer's solution were prepared and the phage concentration estimated simultaneously by all three methods. Full details of these methods have been described elsewhere (7, 8), but if a phage was re-examined for heat resistance the strength was determined before each heat exposure, unless the interval between the two examinations was not greater than 4–7 days.

Considerable variation was observed in the strength of the phages by different tests, but not all strains and their corresponding phages varied in the same way. Two examples of this kind of variation in estimating the phage concentration by the three methods used are given in Table 1.

In general, unless the strains grew very slowly in milk, it may be said that the activity test usually gave the highest concentration of phage and that the estimation of plaques was the least sensitive of the three methods (Table 2).

The final estimation of phage strength was empirical and had regard to all three tests. Naturally the exact assessment varied with the individual phage under test.

Nelson *et al.* (3) have shown that when testing *Str. lactis* cultures against a group of phages by the plaque method and by a litmus-milk-culture method the agreement was by no means perfect. They found that 'in most cases where agreement was lacking, the litmus-milk-culture method indicated the presence of inhibitory principle while the plaque method gave negative results, but the situation was sometimes reversed'.

Table 1

Strain and phage no. ...	295/1			306/12		
Phage dilution	Milk activity after 6 hr.* incubation	Broth test	Plaque formation	Milk activity after 7 hr.* incubation	Broth test	Plaque formation
(1) 1/10	2·1	+	.	2·5	+	.
(2) 1/100	2·1	+	.	2·5 Phaging	+	Innumerable
(3) 1/1000	2·1	+	.	2·6	+	Innumerable
(4) etc.	2·2	+	Innumerable	3·5	+	–
(5)	2·2 Phaging	+	Innumerable	3·5	+	–
(6)	2·5	+	14	3·5	+	–
(7)	2·7	–	4	3·5	+	–
(8)	2·9	–	–	3·5	–	.
(9)	2·8	–	–	3·5	–	.
(10)	4·2	–	.	3·5	–	.
(11)	4·7	–	.	.	.	.
Control	4·1	–	.	3·5	–	.
Estimated phage concentration		10^8			10^6	

* Figure given = ml. of $N/10$ NaOH required to neutralize the acidity of 10 ml. of milk.
. = not tested.

Table 2. *Estimation of bacteriophage concentration by three different methods showing highest dilution in which phage was present*

Phage	(*a*) Activity in milk	(*b*) Clearing of broth	(*c*) Plaque formation	Estimated phage concentration
*y*16	10^{-9}*	10^{-9}	10^{-7}	10^8
156/2	10^{-8}	10^{-8}	†$10^{-(7-10)}$	10^8
161/8	10^{-6}?	10^{-7}	†$10^{-(5-8)}$	10^7
166/7	†$10^{-(1-11)}$, v. slow	10^{-7}	†$10^{-(5-8)}$	10^7
236/9	10^{-7}?, slow	10^{-9}	10^{-7}	10^7
262/3	10^{-3}?, slow	10^{-5}	10^{-5}*	10^5
278/12	10^{-8}	10^{-9}	†$10^{-(3-8)}$	10^8
295/12	10^{-8}	10^{-5}	10^{-5}	10^8
303/12	10^{-9}	10^{-7}	10^{-3}	10^9
219/12	10^{-6}	10^{-5}	.	10^6
295/1	10^{-8}	10^{-10}?	.	10^8
170/7	10^{-7}	10^{-7}	10^{-5}	10^7
174/11	10^{-10}*	10^{-10}*	†$10^{-(2-7)}$	10^{10}
318*b*/27	10^{-9}	10^{-8}*	10^{-5}	10^9
303/4	10^{-5}	10^{-6}*	10^{-4}	10^5
306/12	10^{-3}	10^{-7}*	10^{-3}	10^6
1*p*5	10^{-9}	10^{-9}	10^{-7}*	10^8
144*f*	10^{-9}	10^{-9}	10^{-7}*	10^9
*y*1	10^{-5}	10^{-5}	10^{-7}*	10^5
161/3	†$10^{-(1-10)}$, v. slow	10^{-8}	10^{-7}	10^7
*y*6	10^{-10}*	10^{-9}*	10^{-5}	10^8
156/1	10^{-7}	10^{-9}	10^{-7}*	10^8
223/13*l*	†$10^{-(1-10)}$, v. slow	10^{-9}	10^{-7}*	10^8
223/13*s*	†$10^{-(1-10)}$, slow	10^{-11}*	10^{-7}*	10^{10}
185/1	†$10^{-(1-10)}$, v. slow	10^{-6}	†$10^{-(2-7)}$	10^6
216/9	10^{-6}?, slow	10^{-7}	10^{-6}	10^7

* = phage demonstrated in that dilution but not tested in higher dilutions so that phage may have been present in dilutions higher than given.

† = dilutions which were tested for phage and in which phage was not present. Lower dilutions not tested.

Slow = slow-growing in milk.

Whitehead & Hunter (2) have commented on the fact that a phage having given what appeared to be plaques on agar produced no corresponding activity in liquid media (presumably broth).

Experience with certain phages (including 338/2 and 338/10 which were not used in this work) has shown that they rarely cleared broth but, when tested in milk by the activity test, acid development was completely inhibited.

Variation in the strength of the phage with the medium used to assess it introduces a further variable which makes strict comparisons between the heat resistances of phages difficult.

Determination of heat resistance

The phages were prepared as Seitz (S.B.) filtrates in yeast dextrose broth (Y.D.B.). For the heat-resistance test the phage preparation was added to 5 ml. quantities of sterile separated milk contained in small test-tubes ($5 \times \frac{1}{2}$ in.). The *p*H of the milk, which was always from the same selected cows, varied between 6·42 and 6·28. Phage was introduced by a standardized dropping pipette so that as the original concentration of the phage preparation was known, the titre of the phage in the milk undergoing test could be estimated. The final concentration of phage desired was 10^6, but there was some variation. Immediately before the test-tubes of milk were inoculated they were held in the water-bath at the desired temperature in order to reduce the time lag of the mixture in reaching the temperature of the water in the bath. After the phage had been added sterile rubber bungs were substituted for the cotton plugs in the tubes which were clipped on to a wheel revolving, completely immersed, in a constant-temperature water-bath. At certain time intervals the tubes were removed, cooled at once in running cold water and put in the ice-chest (4–6° C.) and kept there until the next morning.

Tubes containing milk only were used as controls throughout the test. They were exposed to heat in the same way as the test-tubes to which phage had been added and as a rule two tubes were heated at 70° C. for 10 min. for each phage under test. On any one day one phage preparation was used for heat-resistance tests at the various temperatures shown in Table 3 (e.g. test 1). Any one series of observations at the various temperatures and times of exposure was completed within a few hours. Later, heat-resistance tests on the phages were repeated (e.g. test 2, etc.) but the strength obviously was not always the same as in the first test.

Method of demonstration of survival of phage after heat exposure

(*a*) *Using methylene blue as indicator of phaging of culture in milk.* After the suspensions of phage in milk had been heated, 1% of an 18 hr. culture of the streptococcus corresponding to the phage race under test was added. Sterile methylene-blue solution to give an approximate final concentration of 1/200,000 of methylene blue was also added to the tubes, which were placed in a water-bath at 30° C. and examined at intervals for reduction of colour. In those tubes in which phage had failed to survive, the colour was reduced and the milk eventually coagulated. Where phage had survived, the colour either remained unchanged or was reduced initially for a short period. The blue colour, of course, returned as the lysis of the starter organisms proceeded. Some of the strains grew relatively slowly and therefore this method did not yield results within a convenient time, i.e. 8–12 hr. When the strength of the phage was low the organisms might, within the time of the test, retain sufficient vitality to reduce the dye and therefore a result which should have appeared as positive might be reported by this test as negative.

(*b*) *Using yeast dextrose broth and increasing the phage strength by 'feeding'.* A second method was therefore used to determine the survival of phage. Phages surviving heating were strengthened by 'feeding' in three successive subcultures of the corresponding organisms. The original medium in which the phage had been heated was inoculated with 1–3 ml. of a Y.D.B. culture of the appropriate organism and incubated at 30° C. for 5–7 hr. Tubes of Y.D.B. were then inoculated from the incubated milk tubes and placed in the ice-chest until the next day, when $\frac{1}{2}$ ml. of a 1/10 dilution of an 18 hr. culture of the same strain was added and the tubes reincubated and observed at intervals for clearing (i.e. phaging). Those cultures which had not phaged within 7 hr. were subcultured into Y.D.B. and again placed in the ice-chest until next day, when they were inoculated with the test culture and reincubated. If this third test failed to indicate the presence of phage, it was assumed that it had not survived the original heat treatment.

RESULTS AND DISCUSSION

Table 3 shows the range of heat resistance in separated milk of the collection of bacteriophages tested.

(1) It will be observed that the Y.D.B. method of demonstrating the survival of a phage after heat exposure, even allowing for some variation in the titre of the phage suspensions, appears to be the more sensitive of the two methods. This is perhaps to be expected since the method embodies the 'feeding' of a weak phage which, using methylene blue in milk as the indicator, might be missed. Further, if a strain of *Streptococcus* happened to grow slowly in milk the effect of residual phage, even at high titre, might be missed in milk, whereas in broth, in which such strains grow better, a low residual phage titre would be revealed. It is clear, therefore, that the residual titres of some phages after heating will appear to be higher in Y.D.B. than in milk, e.g. 166/7 and 219/12.

(2) Most of the phage races tested withstood exposure to heat at 65–67° C. for 30–35 min., whilst less than half of the races survived the same temperature for 50–60 min.

After heating at 70° C. for 10–15 min. most of the phages survived, and those which did not were mainly races which had been heated in low concentration of 10^4 (per 5 ml. of milk) or lower.

Survivals of phage after heating at 75° C. even for short periods ($2\frac{1}{2}$–$7\frac{1}{2}$ min.) were rare. Thus races of phage attacking lactic streptococci from cheese starters appear to be destroyed by a temperature of 75° C. for 10 min.

In the preparation of milk for bulk starter, for which milk must be freed from phage contamination, the common practice of holding the milk at temperatures from 190 to 212° F. for periods of $\frac{3}{4}$–$1\frac{1}{2}$ hr. is obviously sound, and allows that large margin of safety which is necessary in practice. The desirability of allowing such a margin of safety is emphasized when the high concentration of phage which may be present in cheese factories and the occasional lapses and accidents which are to be expected are taken into consideration.

SUMMARY

The heat resistance, in milk, of bacteriophages attacking strains of lactic streptococci isolated from cheese starters has been determined. Active phages did not usually survive a temperature of 75° C. for $7\frac{1}{2}$ min. but many phages were not destroyed by heating for

Table 3. *Heat resistance of phage*

Determination of survival using (*a*) methylene blue in milk

Phages	1*p*5	144*f*	*y*6	*y*16	156/1	156/2	161/3	170/7	174/11	236/9	278/12	295/1	295/12	303/12	318*b*/27	161/8	168/7	306/12	*y*1	219/12	303/4	262/3
No. of test ...	1	1	.	1	.	.	1	1	1	1	1	1	1	1	1	1	1	1	.	1	.	1
Conc. of phage per 5 ml. milk ...	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^6	3×10^6	10^6	3×10^6	3×10^6	3×10^6	3×10^6	10^6	10^5	10^5	10^5	10^4	3×10^4	10^4	6×10^3
Heat exposure																						
65°C. for 5 min.	+	+	+	.	+	.	+	+	+	+	+	+	+	+	+	.	+	+	+	+	+	+
10 min.	+	+	+	+	+	+	+	+	+	.	+	+	+	+	+	+	.	+	+	+	+	+
15 min.	+	+	+	.	.	.	+	+	.	+	+	+	+	+	+	.	−	+	+	+	+	−
20 min.	.	.	.	.	.	.	.	+	+	.	+	.	−	+	.	.	.	.	.	.	.	.
25 min.	.	.	.	+	.	+	.	.	.	+	.	.	.	.	.	−	.	.	.	.	.	.
30 min.	+	+	.	.	+	.	+	.	.	.	.	+	.	.	+	.	.	+	+	−	.	−
35 min.	.	.	.	.	.	.	.	+	+	+	+	.	−	−	.	.	−	.	.	.	.	.
40 min.	.	.	.	−	.	+	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	−
45 min.	+	+	+	.	+	.	+	.	.	−	.	.	.	.	+	.	.	+	+	.	.	.
50 min.	.	.	.	.	.	.	.	+	+	.	+	−	−	−	.	.	.	.	.	−	−	.
60 min.	+	+	+	.	+	.	+	.	.	−	.	−	.	.	+	.	−	+	+	−	.	−
70°C. for 5 min.	+	+	+	+	+	+?	+	+	+	−	+	−	+	+	+	−	−	+	+	−	−	−
7½ min.	.	.	.	.	.	.	.	−	+	.	+	.	−	.	.	.	.	.	.	.	.	.
10 min.	+	−	+	.	+	.	−	−	+	−	+	−	−	−	−	.	−	+	+	−	−	−
15 min.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	−	.	.	−	.
20 min.	+	−	+	−	+	−	−	+	+	−	+	−	−	−	−	−	−	.	+	−	.	−
30 min.	+	−	−	−	−	−	−	.	.	−	.	−	.	.	−	−	−	−	−	−	−	−
75°C. for 2½ min.	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
5 min.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7½ min.	−	−	−	−	−	.	−	−	−	−	−	−	−	.	−	.	.	−	−	−	−	−
10 min.	.	.	.	−	.	−	.	.	.	.	.	.	.	.	.	−	−	.	.	.	.	.
Control 1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Table 3 (*continued*)

Determination of survival using (*b*) yeast dextrose broth

Phages	223/13*s*	161/3	1*p*5	174/11	1*p*5	144/*f*	*y*16	161/8	216/9	216/9	278/12	1*p*5	170/7	174/11	185/1	223/13/	233/13*s*	236/9	236/9	278/12	303/12	166/7	223/13*s*	236/9	262/3	219/12	318*b*/27	295/12
No. of test ...	3	2	4	2	2	2	2	2	1	2	2	3	2	3	.	.	2	3	4	3	2	2	1	2	2	2	2	2
Conc. of phage per 5 ml. milk ...	10^9	10^8	10^7	10^7	10^6	10^6	10^6	10^6	10^6	10^6	10^6	10^5	10^5	10^5	10^5	10^5	10^5	10^5	10^5	10^5	10^5	10^4	10^4	10^4	10^4	10^3	10^3	10^3
Heat exposure		√					√	√																√				
67°C. for 5 min.	.	+	.	+	+	+	+	+	+	+	+	.	+	.	+	+	.	.	.	.	+	+	+	+	+	+	+	+
10 min.	.	−	.	+	+	+	+	+	+	+	+	.	+	.	+	−	.	.	.	.	+	+	+	+	+	+	+	−
15 min.	.	+	.	+	+	+	+	+	+	.	+	.	+	.	+	+	.	.	.	.	+	+	+	→	+	+	+	−
30 min.	.	+	.	+	.	+	−	−	+	+	+	.	+	.	−	.	.	.	.	.	−	+	+	−	+	−	+	−
40 min.	.	−	.	.	.	−	.	.	.	.	.	.	.	.	.	−	.	.	.	.	.	.	.	.	.	.	.	.
45 min.	.	.	.	+	+	.	−	−	+	+	+	.	+	.	−	.	.	.	.	.	−	.	.	−	−	−	+	−
60 min.	.	−	.	+	−	−	−	−	+	+	+	.	+	.	−	−	.	.	.	.	−	−	+	−	−	−	−	−
75 min.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
70°C. for 5 min.	+	+	.	+	+	+	+	+	+	+	+	.	+	.	+	+	+	+	+	.	−	+	+	+	+	+	+	−
7½ min.	.	+	.	+	+	+	+	−	+	+	+	.	+	.	+	+	+	+	.	.	−	+	+	−	+	+	+	−
10 min.	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	.	−	+	−	−	+	+	−	−
12½ min.	.	.	.	+	.	.	.	−	+	+	+	.	.	.	+	.	.	.	.	.	−	+	−	−	.	−	.	−
15 min.	+	−	+	+	+	−	−	−	+	+	+	+	+	+	−	+	+	+	+	+	−	+	−	−	−	−	−	−
30 min.	−	.	−	+	−	.	.	.	+	−	+	+	−	+	−	.	+	−	−	−	−	−	.	−	−	−	−	−
40 min.	+	.	−	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	−	.	.	.	.	.	.	.	.
75°C. for 2½ min.	−	−	−	+	−	−	−	−	−	+	+	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
5 min.	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	−	−	−	−	.	−	−	−	−
7½ min.	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
10 min.	−	.	.	.	.	.	.	.	.	.	.	−	.	−	.	.	.	.	−	.	.	.	.	.	.	.	.	.
Control 1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

+ =survival of phage demonstrated. − =survival of phage not demonstrated. . =no test.

50–60 min. at 65–67° C. and most survived at 70° C. for 10–15 min. The practical implications of these findings in the 'sterilization' of milk for starter making have been discussed. Growth in yeast dextrose broth was found to be superior to methylene blue reduction for the demonstration of residual phages after heating.

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325. A STUDY OF THE BALANCE OF CERTAIN MILK NUTRIENTS IN THE MAKING OF CHEDDAR, CHESHIRE AND STILTON CHEESES, AND OF THEIR FATE DURING THE RIPENING OF THE CHEESES

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(With 3 Figures)

Several reports have been published dealing with the recovery in cheese of various major components of milk and the comprehensive study of McDowall(1), which contains numerous references to earlier work, may be quoted as an example.

There is less information about the fate of 'minor' constituents such as vitamins, and also of some minerals. For this reason the study now described was undertaken in the spring and summer of 1940.

Three typical English cheeses were made, Cheshire and Cheddar, the two most popular hard varieties, and Stilton, the characteristic blue-veined cheese. All three were made twice during one season, in March and June, that is, at times when the differences in the composition of milk are most pronounced. On both dates a common bulk of milk was used. It should be realized that Stilton would not usually be made in March, and that in general in the planning of this study a certain compromise had to be achieved between experimental requirements and normal cheese-making practice.

EXPERIMENTAL

(1) *Source of milk*

In March and June the mixed raw milk from morning and evening milkings of two consecutive days was obtained from the same farms in the neighbourhood of Reading. The herds comprised Shorthorns, Friesians, Ayrshires and Guernseys. On both occasions the milk was bulked and then divided between the three varieties of cheese.

(2) *Preparation and sampling of the cheese*

The cheese were made in the experimental dairy of the Institute. The methods of manufacture were those generally adopted in this country as outlined, for example, in a bulletin of the Ministry of Agriculture and Fisheries (2) but adapted to the local conditions. No colouring matter was used so as not to complicate the determination of carotene. Each bulk of cheese was made from about 460 kg. of milk; of the hard varieties two 20 kg. cheese and a small one were made each time; the yield of Stilton was seven 6 kg. cheese and a sample one, at each making. Of Cheddar and Cheshire one large cheese was used for sampling for chemical tests at approximately monthly intervals during the ripening period. Portions of the cheese representative of the whole were taken each time, according to a predetermined sampling chart, by means of a cylindrical borer. The spaces left were immediately filled and sealed with paraffin-wax candles. Owing to their nature, Stiltons could not be sampled in this way and a fresh cheese was taken each month. The remaining winter Cheddar and Cheshire cheeses were used for biological tests. Calculations are based on actual weighings with the exception of the weight of the first curd which could not be obtained directly. It was derived for each cheese from the combined weights of the second whey and curd. The measured weight of the first whey was also corrected for unavoidable spillage. Weights of cheese quoted are fully compensated for all samples removed during ripening.

The stages of cheese-making at which sampling took place and the time intervals elapsing between these stages are given in Table 1.

Table 1. *Scheme of sampling*

Description of sample	Time interval from start of cheese-making: March	June	March	June
	Cheddar		Cheshire	
(*a*) Cheddar and Cheshire				
Milk, raw bulked from evening and morning milkings during 2 days	0	0	0	0
1st whey, 1st curd } bulk at whey drawing	3½–5 hr.	3–4 hr.	4–4½ hr.	2¾–3½ hr.
2nd whey, during drying of curd, 2nd curd, after milling, before salting }	6½–8½ hr.	5½–6½ hr.	6–6½ hr.	4¾–5½ hr.
3rd whey, from press, 3rd curd, pressed (24 hr. old cheese) }	24 hr.	24 hr.	24 hr.	24 hr.
(*b*) Stilton	Stilton			
Milk, as for Cheddar and Cheshire	0	0		
1st whey, 1st curd } after turning out of cloths	16 hr.	14 hr.		
2nd whey, during drying, 2nd curd, before salting }	23½ hr.	21 hr.		
3rd whey, drainings from hoop, 3rd curd, 24 hr. old cheese }	48 hr.	51 hr.		
4th whey, drainings, hooping to scraping	1 week	1 week		

Regarding quality, both winter- and summer-made Cheddar and Cheshire were marketable cheese of good keeping quality. The Stilton were of good flavour but lacked mellowness.

(3) *Nutritive values of the cheese as determined by chemical assay*

(*a*) *Analytical methods*

Fat was measured by the Gerber test as described by the British Standards Institution (3), moisture by drying in a steam oven to constant weight. Vitamin A and carotene were measured in milks and wheys in the fat prepared by extraction or by churning. In both instances the methods were those described by Gillam, Henry, Kon & White (4), but for extracting wheys the quantity of all reagents was reduced by half. Curds were ground with four times their weight of water and the mixture extracted as if it was milk. Carotenoids and vitamin A were measured in a Lovibond tintometer calibrated against pure β-carotene and a solution of vitamin A of known potency, results being expressed in μg. and i.u. respectively.

Vitamin B_1 and riboflavin in milks and wheys were measured as described by Houston, Kon & Thompson (5). For the estimation of vitamin B_1 in curds these were finely ground with three times their weight of water, pepsin (Parke, Davis and Co.) was added to the extent of 1/40th of the weight of the curd, the *p*H adjusted to 2 and the mixture incubated for 48 hr. at 37° C., the same amount of pepsin being added after the first 24 hr. The *p*H was then adjusted to 4 and the volume made to 100 ml. The subsequent treatment, including incubation with takadiastase, was as for milk and whey. For riboflavin measurements 30 ml. of 75% v/v methanol were added to 20 g. of finely divided curd and the mixture kept in darkness for 24 hr. at 37° C. It was then thoroughly ground in a mortar, made up to 80 ml. with warm distilled water and held for 20 min. at 60° C. After cooling, 0·1 ml. of glacial acetic acid was added, and the mixture shaken and filtered. The filtrate was successively treated with 0·5 ml. of $N/10$ $KMnO_4$ and 1 ml. 3% H_2O_2 and the fluorescence of a 10 ml. aliquot read as for milk.

Vitamin C was measured in milk and wheys as described by Henry, Houston, Kon & Thompson (6). Twenty-gram samples of curd were ground with 20 ml. of 10% sodium hexametaphosphate (6), 20 ml. of distilled water and 0·5 ml. of conc. HCl. After further grinding the mixture was filtered and the filtrate treated as milk. The titration of curds was difficult because of an indistinct end-point.

Before presenting the results it might be well to point out some sources of error inherent in a balance experiment of this kind. In the first instance most analyses were done of necessity on samples representing only a small fraction of the total bulk, and the calculation of total quantities involved, therefore, the use of large factors which magnify normal inaccuracies. Again, the nature of certain products such as early curds rendered satisfactory sampling, especially for the estimation of fat, extremely difficult. In whey, the estimation of the small amounts of fat present is a notoriously hazardous analytical task. Finally, the uncertainties of the chemical assay of vitamins which are rather greater than those of more straightforward chemical tests superimposed their errors on those already mentioned. The minute details of the picture obtained in these studies may therefore be somewhat distorted, but there is reason to believe that the general outline is true.

(*b*) *Results*

(i) *Water-soluble vitamins.* All analytical data are given in Table 2. The balances of water-soluble vitamins during cheese-making, and their subsequent fate during ripening, are shown as histograms in Fig. 1. The white portion in each histogram represents the

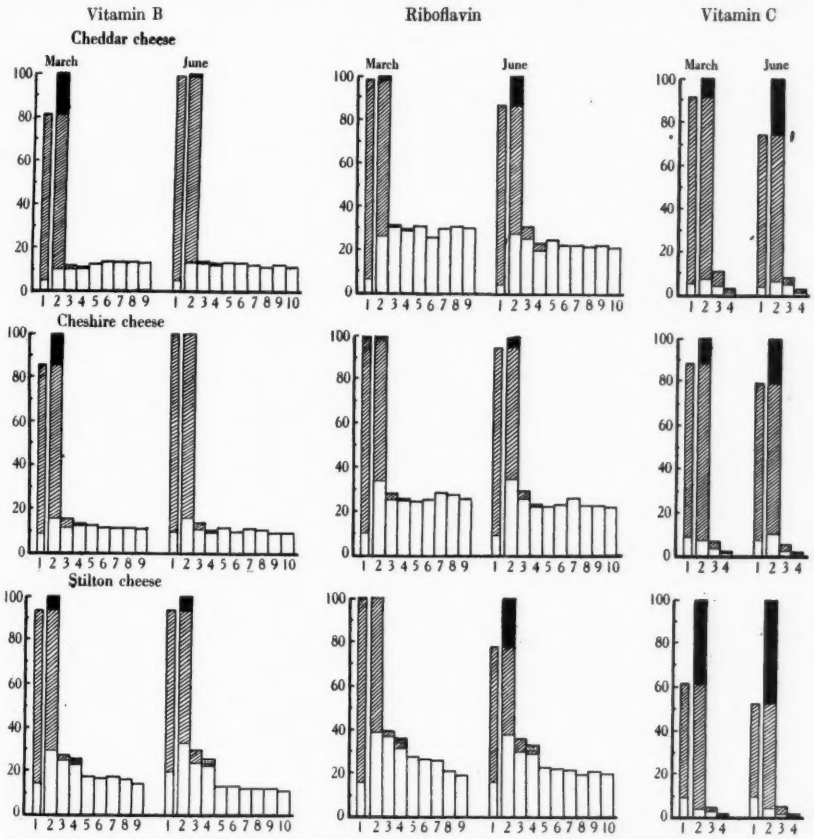

Fig. 1. Percentage distribution of water-soluble vitamins between milk, curd, whey and cheese.

□ = percentage of vitamin present in milk found in curd or cheese.

▨ = percentage of vitamin present in milk found in whey.

▩ = percentage of vitamin present in milk found in Stilton scrapings and 4th whey.

■ = percentage of vitamin present in milk not accounted for.

Key to Fig. 1. Column 1 = percentage distribution of vitamin between the 1st curd and whey that would obtain if the vitamin were only present in the aqueous phase. Column 2 = percentage recovery of the vitamin, originally present in milk, in 1st curd and 1st whey. Column 3 = percentage recovery of the vitamin, originally present in milk, in 2nd curd and 2nd whey. Column 4 = percentage recovery of the vitamin, originally present in milk, in 3rd curd and 3rd whey, and also, for Stilton cheese, in scrapings and 4th whey. Column 5 = percentage recovery of the vitamin, originally present in milk, in cheese sample 1.* Column 6 = percentage recovery of the vitamin, originally present in milk, in cheese sample 2.* Column 7 = percentage recovery of the vitamin, originally present in milk, in cheese sample 3.* Column 8 = percentage recovery of the vitamin, originally present in milk, in cheese sample 4.* Column 9 = percentage recovery of the vitamin, originally present in milk, in cheese sample 5.* Column 10 = percentage recovery of the vitamin, originally present in milk, in cheese sample 6.*

* See Table 2.

amount of a given vitamin in curd or cheese, the shaded portion refers similarly to the vitamin in whey, and the black portion to that part of the original vitamin content of milk not accounted for in the first curd or the first whey. The separate slender column on the left of each histogram shows the distribution of the vitamin between curd and whey that would obtain if it were present only in the aqueous phases of these two fractions.

Vitamin B_1. With the two hard cheeses Cheddar and Cheshire, the main loss of vitamin B_1 took place at the drawing of the first whey. This contained some 70–85% of the total present in the milk; only 10–14% was recovered in the first Cheddar curd and only

Table 2. *Analytical data for milks, curds, wheys and cheeses*

Description of sample	Total wt. of sample kg.			% fat			% total solids			Vitamin A i.u./g. fat		
	Cheddar	Cheshire	Stilton	Cheddar	Cheshire	Stilton	Cheddar	Cheshire	Stilton	Cheddar	Cheshire	Stilton
(*a*) March experiment												
Milk	465·8	461·9	461·6		3·47			12·10			15·2*	
1st whey	414·2	392·4	362·8	0·27	0·33	0·08	6·85	6·85	6·79	16·5	14·4	13·7
2nd whey	6·8	17·6	14·9	0·61	0·22	0·16	7·46	6·82	7·35	16·4	11·5	10·9
3rd whey	2·3	5·6	14·8	3·25	2·25	0·06	20·53	13·80	11·61	14·4	13·9	—†
4th whey	—	—	15·7	—	—	0·04	—	—	17·06	—	—	—†
1st curd	51·6	69·5	98·8	27·0	20·5	17·5	51·4	40·8	35·4	—	12·0	13·3
2nd curd	44·7	51·9	83·9	31·5	27·5	18·5	59·7	54·3	40·1	13·3	13·3	13·5
3rd curd	42·4	46·3	70·9	32·5	31·8	20·8	64·1	61·3	45·5	13·3	13·5	13·3
Scrapings	—	—	0·6	—	—	20·5	—	—	40·9	—	—	—
Cheese:												
1st sample,‡ 6 weeks old	40·7	44·1	47·0	34·0	31·6	32·8	65·6	59·6	58·3	13·3	13·3	13·3
2nd sample,‡ 11 weeks old	39·9	43·0	41·2	34·5	32·5	34·9	66·2	62·8	64·9	13·3	13·3	13·3
3rd sample,‡ 17 weeks old	39·5	42·2	37·2	34·5	32·9	37·9	67·0	63·3	68·4	12·8	12·8	12·0
4th sample,‡ 24 weeks old	39·2	39·7	35·3	34·8	33·9	40·0	67·3	64·5	71·3	12·5	12·5	12·5
5th sample,‡ 37 weeks old	38·5	38·2	32·7	36·6	35·5	43·5	69·4	67·3	74·6	14·5	13·5	15·0
(*b*) June experiment												
Milk	462·4	456·5	451·7		3·60			12·28			27·9* / 27·5	
1st whey	411·8	382·2	334·1	—	—	—	6·88	6·76	6·82	25·0*	26·8*	22·5* / 20·8
2nd whey	3·4	23·2	37·8	—	—	—	7·42	6·82	7·47	24·0*	23·7*	19·0*
3rd whey	2·4	4·4	12·4	—	—	—	41·45	17·79	11·61	25·0	26·8	19·3
4th whey	—	—	15·3	—	—	—	—	—	11·27	—	—	13·9
1st curd	50·6	74·3	117·6	30·3	25·4	17·9	58·4	48·5	31·4	27·5	26·8	26·8
2nd curd	47·2	51·1	79·8	30·6	28·8	18·6	58·5	56·2	41·8	28·8	27·8	26·8
3rd curd	45·1	46·7	69·0	30·9	30·2	23·3	61·7	59·0	44·0	27·5	26·8	23·8
Scrapings	—	—	0·7	—	—	19·7	—	—	58·3	—	—	—
Cheese:												
1st sample,‡ 7 weeks old	42·5	44·0	40·7	32·2	32·8	35·5	63·0	62·9	63·3	25·0	22·1	24·0
2nd sample,‡ 13 weeks old	41·6	43·0	39·0	33·5	33·6	37·9	64·4	64·2	66·7	23·4	23·4	24·0
3rd sample,‡ 19 weeks old	40·9	42·2	35·1	35·4	34·9	43·0	65·6	65·1	73·2	26·8	21·2	26·8
4th sample,‡ 25 weeks old	40·4	41·7	33·3	34·8	34·0	44·0	66·1	65·4	77·1	25·0	25·0	25·0
5th sample,‡ 34 weeks old	39·8	40·9	31·2	35·2	34·7	46·4	66·5	65·8	79·3	25·6	25·0	25·6
6th sample,‡ 42 weeks old	38·7	39·9	29·8	35·6	35·2	46·7	67·1	67·2	81·1	23·4	23·4	23·4

* These estimations were done on fat obtained by churning, other vitamin A and carotenoid estimations were done on extracted fat, cf. pp. 102, 107.

† Too weak to read.

‡ Samples taken from the same cheese for Cheddar and Cheshire, for Stilton a fresh cheese was used at each sampling, cf. p. 101.

16% in the first Cheshire curd. In the winter experiment 15–20% of the quantity originally present in milk could not be accounted for, but in summer the balance tallied very much better. The loss of vitamin B_1 in the second and third Cheddar wheys was very small, less indeed than the experimental error of these balance measurements. As would be expected the drainage from the Cheshire curd was more marked, with the result that the recoveries of vitamin B_1 in the young cheeses of both varieties were essentially the same, 13% for Cheddar and 12% for Cheshire. No significant losses occurred during ripening, though this was allowed to continue for longer than the usual practice. There was a tendency, however, for slightly lower values in the Cheshire cheese, even when allowance was made for its higher moisture content. It is problematical whether these

differences are real. The ripe Cheddar cheese contained 53–57, the ripe Cheshire 43–46 μg. vitamin B_1 per 100 g. The values for Cheddar agree well with those obtained in the United States for the American variety (7, 8), but are much higher than Pyke's (9) value of 12 μg./100 g. obtained in this country. For Cheshire the agreement with Pyke's figure of 33 μg./100 g. is closer. In view of the similarity of manufacture of these two kinds of cheese the differences reported by Pyke are surprising.

In the manufacture of Stilton cheese a smaller proportion of vitamin B_1, some 60%, passed into the first whey and a correspondingly large amount, about 30%, was re-

Table 2 (*continued*)

Carotenoids μg./g. fat			Vitamin B_1 μg./100 g.			Riboflavin μg./100 g.			Vitamin C mg./100 g.		
Cheddar	Cheshire	Stilton	Cheddar	Cheshire	Stilton	Cheddar	Cheshire	Stilton	Cheddar	Cheshire	Stilton
	1·2*			33·5			84·4			1·58	
5·53	3·64	11·40	27·0	27·8	27·6	68·3	65·7	66·7	1·49	1·51	1·15
2·92	4·15	7·42	26·1	27·4	18·7	63·3	64·1	61·7	0·67	1·15	0·71
2·83	2·43	7·47	16·5	16·9	14·8	68·6	64·1	61·2	0·47	0·43	0·38
—	—	25·8	—	—	16·8	—	—	69·9	—	—	0·17
—	2·12	2·02	29·6	34·2	45·2	199·0	190·0	152·0	1·01	0·73	0·32
2·56	2·08	2·10	36·2	34·2	45·8	268·0	191·0	171·0	0·73	0·54	0·29
2·02	2·00	2·09	40·0	40·0	50·0	268·0	212·0	172·0	0·30	0·24	0·07
—	—	—	—	—	49·0	—	—	154·0	—	—	0·07
2·04	2·04	1·98	50·0	43·0	58·0	297·0	220·0	229·0	—	—	—
1·98	1·96	1·98	53·0	43·0	63·0	252·0	232·0	244·0	—	—	—
2·00	1·96	1·88	54·0	43·0	80·0	295·0	264·0	267·0	—	—	—
1·94	1·88	1·88	53·0	45·0	80·0	308·0	278·0	226·0	—	—	—
2·00	2·00	2·10	53·0	45·0	80·0	306·0	264·0	226·0	—	—	—
	10·1* 11·2			42·3			128·0			1·71	
9·7*	10·1*	9·2* 18·0	40·2	42·6	34·6	84·7	90·8	69·2	1·30	1·30	1·03
10·2*	9·9*	7·2*	32·3	25·9	28·5	83·7	89·9	84·7	0·68	0·85	0·73
9·2	9·7	7·9	25·8	26·9	16·7	91·2	110·8	63·9	0·45	0·48	0·48
—	—	33·3	—	—	16·8	—	—	55·9	—	—	0·49
10·2	10·1	10·1	53·3	41·7	53·3	324·0	281·0	184·0	0·99	0·99	0·30
10·4	10·0	10·1	53·3	40·8	56·7	325·0	297·0	216·0	0·84	0·42	0·12
10·6	10·1	10·1	53·3	40·8	60·4	264·0	282·0	245·0	0·24	0·23	0·04
—	—	—	—	—	62·5	—	—	281·0	—	—	0·04
8·6	9·0	8·3	60·0	53·0	65·0	343·0	296·0	315·0	—	—	—
9·3	9·3	9·2	60·0	45·0	67·0	317·0	321·0	323·0	—	—	—
9·8	8·6	9·7	55·0	49·0	68·0	323·0	366·0	345·0	—	—	—
9·8	9·9	9·9	53·0	48·0	73·0	322·0	322·0	333·0	—	—	—
8·8	8·8	9·0	57·0	45·0	76·0	336·0	330·0	380·0	—	—	—
8·2	7·6	8·4	57·0	46·0	75·0	330·0	330·0	375·0	—	—	—

* These estimations were done on fat obtained by churning, other vitamin A and carotenoid estimations were done on extracted fat, cf. pp. 102, 107. † Too weak to read.

‡ Samples taken from the same cheese for Cheddar and Cheshire, for Stilton a fresh cheese was used at each sampling, cf. p. 101.

covered in the curd. The unaccountable losses were the same with winter and summer milk and amounted to 6–7%. At further stages of manufacture there was a gradual loss of vitamin B_1 from the curd not fully accounted for by drainage in the whey, but little if any loss took place during the ripening of the actual cheese. The proportion of the original vitamin B_1 retained in the young cheese was 17% in winter and 14% in summer, i.e. slightly more than in the hard varieties. In the ripe cheese vitamin B_1 amounted to 70–80 μg./100 g., a higher value than for Cheddar or Cheshire. Pyke (9) found only 24 μg./100 g. in Stilton cheese.

As is evident from a comparison of the first and second columns in the histograms, in all three cheeses the amount of vitamin B_1 retained in the curd was appreciably greater

than would be expected if the vitamin was wholly present in the aqueous phase. The difference between the vitamin B_1 values for the curd in columns 1 and 2 represents that fraction of the vitamin B_1 of milk combined with the curd solids. Details are given in Table 3. It will be seen that the percentage of bound vitamin B_1 corresponds for Cheddar and Cheshire with the proportion of vitamin B_1 in firm combination with protein found for liquid milk in this laboratory (5). For Stilton the values are higher, indicating that the curd had also possibly retained some of the vitamin B_1 loosely combined with protein. It will be seen from the table that vitamin B_1 in a combined form constitutes about one-half of the total quantity of this vitamin in the curd; in other words, if vitamin B_1 was only present in the milk uncombined with other substances the concentration in cheese would be reduced by one-half.

Riboflavin. In winter the main loss, as with vitamin B_1, again occurred in the first whey and amounted to 72% for Cheddar and 66% for Cheshire. The unaccountable loss was negligible, and the percentage of riboflavin recovered in the curd, 26 and 34% respectively, was rather more than double that of vitamin B_1. In summer the proportion retained in the curd was similar, but less riboflavin passed into the whey, as unaccountable losses were more appreciable. It seems reasonable to suggest that these may have been

Table 3. *Percentage of the vitamin B_1 and riboflavin of milk present in the curd in a combined form*

	Vitamin B_1				Riboflavin			
	March		June		March		June	
	*a**	*b**	*a**	*b**	*a**	*b**	*a**	*b**
Cheddar	4·5	46·9	8·7	63·0	20·1	76·7	23·1	83·4
Cheshire	7·1	45·8	6·3	39·1	23·8	70·4	26·6	74·3
Stilton	14·2	49·0	13·6	41·2	22·6	58·7	21·5	57·5

* a = combined vitamin B_1 or riboflavin expressed as a percentage of the total in milk. b = combined vitamin B_1 or riboflavin expressed as a percentage of that present in curd.

due to the action of the strong summer light. Some further loss occurred during the manufacture of the Cheshire cheese, but throughout ripening there was no loss in either Cheshire or Cheddar. The concentration of riboflavin in the ripe Cheddar and Cheshire was 270 and 300 μg./100 g. in winter and about 330 μg./100 g. for both cheeses in summer. It will be seen that, owing to rather greater losses during manufacture in summer, the greater richness of the summer milk (128 as against 84 μg./100 ml. in winter) had only a slight effect on the concentration in the cheese. Munsell (10) quotes a value of 545–600 μg./100 g. obtained by biological assay for American full cream Cheddar, while Strong & Carpenter (11) and Sullivan, Bloom & Jarmol (12) found for 'American cheese' 660 and 410 μg./100 g. respectively by microbiological tests. Sullivan *et al.* (12) reported also a value of 590 μg./100 g. for a sample of immature 'American cheese' fresh from press. It should be noted that milk in the United States is generally richer in riboflavin than the milks used in this experiment (13).

In the manufacture of Stilton cheese a large unaccountable loss of riboflavin was also observed in summer; in winter the recovery in the first curd and whey was theoretical. Though riboflavin was mainly drained away in the first whey, further losses in manufacture were, as would be expected, more accentuated than in the hard cheeses. Moreover, there also seemed to be some loss during ripening, especially noticeable in the

winter-made cheese. In the ripe cheese the riboflavin was of the same order as in the Cheddar and Cheshire, about 250μg./100 g. in winter and 350μg./100 g. in summer.

The striking feature of all three cheeses was the much greater retention of riboflavin in the curd than would be expected if the factor was entirely present in true solution. The winter Cheshire first curd, for example, contained 34% of the milk riboflavin but only 10% of the milk water. The third curd still had 25% of the milk riboflavin but only 4·4% of the water, and at this stage loss of moisture by drying was as yet of no consequence. The percentages of the total riboflavin of milk thus combined with the cheese solids are given in Table 3; they are uniform for the three varieties and amount to over 20%. The flavoprotein of milk (14, 15, 16, 17, 18) doubtless forms part of this combination. This firmer complex accounts, however, for probably not more than 10% of the milk riboflavin (cf. (18, 19)). The remainder is probably present in a looser combination with casein (cf. (18)). A calculation similar to that carried out for vitamin B_1 (cf. p. 106), shows that the concentration of riboflavin in Cheddar and Cheshire cheeses is about three times greater than it would be if the factor were not partly present in combination with coagulable solids; for Stilton, however, the percentage appears lower because of the greater amount of whey retained in the first curd.

Vitamin C. Vitamin C was rapidly lost from the curds in the process of cheese-making and none of it survived to the ripening stage. The largest loss was due to drainage in the first whey. In winter over 80% of the amount originally present in milk was lost in this way in the making of Cheddar and Cheshire. In summer the quantity was smaller, about 70%, because the unaccounted loss, no doubt due to the action of light, was greater at this season. The amount recovered in the first curd was less than 10% of the original value. This was further rapidly decreased by removal in subsequent wheys and by unaccounted loss, until only less than 2% was found in the third curd; in view of the difficulties of titration, even this figure is probably too high. No measurements were attempted on the cheese.

It is evident from Fig. 1 that the amount of vitamin C found in the first curds was no more than would be expected from the distribution of water between whey and curd.

The findings with Stilton were essentially the same as for Cheddar and Cheshire with the only exception that the longer drainage of the first whey was accompanied by a greater unaccounted loss.

(ii) *Fat-soluble vitamins, vitamin A and carotenoids.* As already mentioned on p. 102 these vitamins were measured in the fat obtained during cheese-making from the various milk fractions, and the calculation of total vitamin yields was based on the determination of the fat content of the different fractions. This measurement proved difficult in some instances, especially in the earlier curds, with the result that balances of distribution of fat, and consequently vitamin A and carotenoids, between milk, wheys and curds are rather erratic. As a further complication, and owing to an unfortunate omission, the fat contents of the summer wheys were not measured.

Some carotenoid estimations on the wheys are now also known to be faulty. It was shown by Henry, Kon, Gillam & White (20) that the difference in carotenoid content between fat extracted by solvents or churned from whole liquid milk was very small and for that reason churning and extraction were both used in this work for obtaining fat from wheys. We now know that this procedure was unjustifiable and that with separated milk or with whey the differences in carotenoid concentration between the

two types of fat may be profound. The differences were much less marked for vitamin A. This problem is discussed in a separate publication(21); the magnitude of differences may be seen from Table 2 where for the June first Stilton whey both values are given. A comparison of values for March wheys, from which all fats were extracted, with values for June wheys which (with the exception of two Stilton ones) were churned, also illustrates the point.

In view of these difficulties we prefer not to give balances for fat, vitamin A and carotenoids in the earlier stages of cheese-making but to quote, as more reliable, concentrations of the latter two substances per gram of fat. If it is wished, the balances can be calculated from data in Table 2. In the March experiment the fat was obtained from all wheys by solvent extraction and the variations in concentration of the carotenoids in these wheys as compared with the concentration in milk are evident. The effect is most marked with Stilton cheese. In the summer experiment measurements of carotenoids made on fat churned from wheys was not substantially different from the value obtained for the original milk, though for Stilton the differences were greater. When, however, extraction was used, the phenomenon observed with the winter cheese was again evident; thus, for the first Stilton whey measurement on the extracted fat gave a value of 18·0 and on the churned fat of 9·2μg./g.

As already mentioned, changes in the method of extraction had little effect on the vitamin A values for wheys and it will be seen that for the hard cheese the concentration of vitamin A in the whey fat was very similar to that in the original milk fat. In Stilton wheys there was progressive loss of vitamin A, probably through oxidation.

On the whole, the curd fat had very much the same concentration of vitamin A and carotenoids as the milk fat, though some slight loss of vitamin A could be noticed in the winter experiment.

There was very little change in the concentration of vitamin A or carotenoids in the fat during the ripening of the cheeses. This is in agreement with the findings of Davies & Moore(22) who found that the vitamin A potency of English Cheddar was of the order that would be expected from its milk fat content. Guerrant, Dutcher & Dahle(23), however, observed a measurable loss of vitamin A potency of Cheddar cheese during ripening, almost wholly compensated for by the progressive loss of moisture of the cheese. In view of the marked fluctuations in the vitamin A and carotenoid content of milk, the significance of comparisons of values obtained by us for Cheddar, Cheshire, and Stilton with those of other authors is rather doubtful. It may, however, be of interest to recalculate our findings in terms of total vitamin A activity by converting μg. of carotenoids into international units on the basis of 0·6μg. per unit. Thus 5 months old Cheddar, Cheshire and Stilton cheeses made from winter milk contained respectively 5·5, 5·3 and 6·2 i.u./g. cheese, while for the summer-made cheeses the figures were 14·4, 14·1 and 18·5 i.u./g. respectively. The higher values for Stilton were due to its lower content of moisture. Davies & Moore(22) quote 7·5 i.u./g. for a sample of English Cheddar, Hathaway & Davis(24) 18·1–24·0 i.u./g. for American Cheddar, and Booher & Marsh(25) 12·0 i.u./g. for the same type of cheese.

Difficulties of calculating total balances of the fat-soluble vitamins during earlier stages of cheese-making have been described above. In later stages the problem became simpler and the recovery of fat and vitamin A and carotene in the first cheeses can be calculated with much better accuracy, since in the estimation of fat at this stage

errors of sampling and analysis are much smaller. These values and those observed during ripening are shown in Fig. 2. The recovery of fat, about 85–90%, is very similar to that observed by workers in this country (26), but is rather less than in New Zealand (1). The recovery of vitamin A and carotenoids follows, on the whole, that of fat, and it will be seen that, as would be expected, the retention amounts to rather more than 80% for

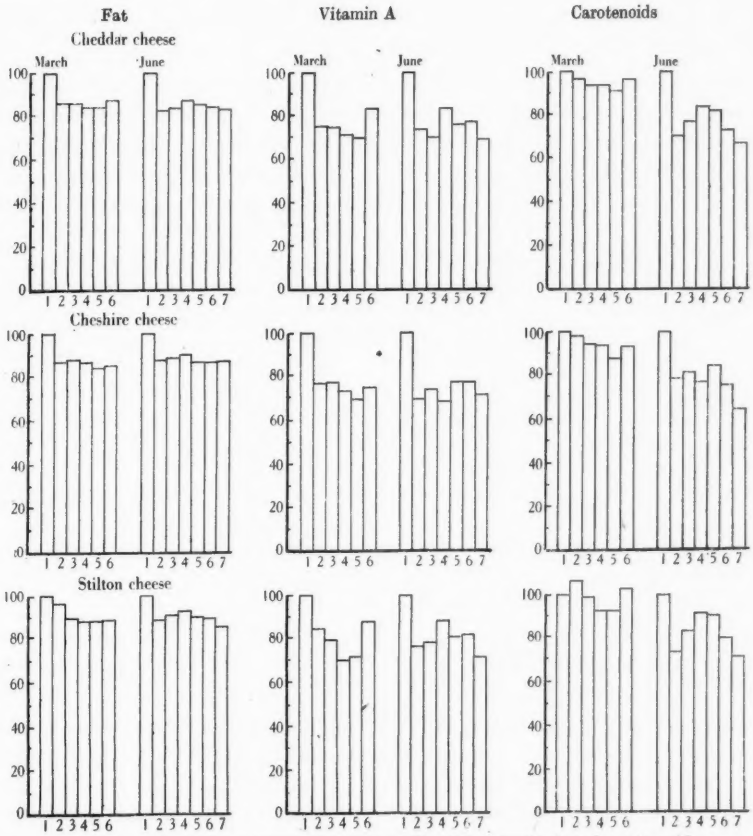

Fig. 2. Percentage recovery in cheese of the fat, vitamin A and carotenoids originally present in milk.

Key to Fig. 2. Column 1 = milk. Column 2 = cheese sample 1.* Column 3 = cheese sample 2.* Column 4 = cheese sample 3.* Column 5 = cheese sample 4.* Column 6 = cheese sample 5.* Column 7 = cheese sample 6.*

* See Table 2.

carotene and rather less than 80% for vitamin A. These recoveries are some three times better than the recovery of riboflavin and some five times better than for vitamin B_1.

(4) *Nutritive values of the cheeses as determined by biological assay*

These experiments were carried out with Cheddar, Cheshire and Stilton cheeses made from winter milk in March 1940 (cf. p. 101). Two experiments were done; in the first the biological value and true digestibility of the proteins of the cheeses were determined;

in the second the growth-promoting value for rats of the cheeses supplemented with whey was compared with that of full-cream spray-dried milk.

For feeding, the cheeses were grated in a household grater, spread on trays and dried at room temperature in a current of air until the moisture content was constant at 5–6%; this was accomplished in 3 days.

(*a*) *The biological value and true digestibility of the proteins of Cheddar, Cheshire and Stilton cheeses*

The biological value and true digestibility of the Cheddar, Cheshire and Stilton cheeses were determined by the method of Mitchell (27, 28). The technique used has been described in detail by Henry, Kon & Watson (29). The cheeses were fed at an 8% level of protein intake (N × 6·38). After making allowance for the fat content of the cheeses, sufficient margarine fat was added to each diet to bring the total fat content to 15%. Table 4

Table 4. *Composition and analysis of the diets used in the nitrogen metabolism experiment*

	Type of cheese		
Component	Cheddar	Cheshire	Stilton
Cheese	22·03	22·77	22·93
Margarine fat	4·43	4·18	2·72
Rice starch	47·54	47·05	48·35
Potato starch	10·00	10·00	10·00
Sugar	12·00	12·00	12·00
Salts*	4·00	4·00	4·00
% N	1·34	1·33	1·29
% moisture	9·93	10·09	9·63

* de Loureiro (30).

gives the composition and analysis of the experimental cheese diets. Four groups of three litter-mate male rats aged 27 days and weighing 59–63 g. were used. Each animal received the different cheese diets in turn in the course of the experiment. The results of the experiment are given in Tables 5–7. It will be seen from Table 6 that for all three cheeses studied very similar values were obtained for the biological values and for the true digestibilities, while Table 7 shows that the small differences observed between the cheeses were without statistical significance. The values of 71–72 for the biological value and 97–98 for the true digestibilities of the three cheeses are very similar to the values of 73 and 98 respectively obtained with Swiss cheese by Beadles, Quisenberry, Nakamura & Mitchell (31) at an 8% level of protein intake. Frengley & Herrick (32), feeding at a 13% level of protein intake, obtained for New Zealand raw milk cheese a biological value of 69 and a true digestibility of 92·5. Using the same rats, they found a value of 64 for casein at a 10% level of protein intake. Our values and those of Beadles *et al.* (31) for the cheese are close to that, 75, obtained for casein by Mitchell (27) and by Henry & Kon (33), and differ in this respect from the findings of Frengley & Herrick (32). These last authors have also compared casein with cheese protein by the growth method of Osborne, Mendel & Ferry (34), at three levels of protein intake and found that the cheese protein gave uniformly better gains in weight per gram protein ingested. They ascribe this superiority to the presence of lactalbumin in cheese. According to Van Slyke & Price (35) lactalbumin in cheese amounts to only about 2% of the casein and it seems doubtful whether any marked supplementary effects could be noted with such a slight admixture.

Table 5. *Individual nitrogen metabolism data for 6-day periods*

Rat no.	Cheese tested	Weight of rat (g.) Initial	Weight of rat (g.) Final	Diet intake g.	N intake mg.	Faecal N mg.	Urinary N mg.	Biological value	True digestibility
				Low egg-nitrogen period					
1	—	60	64	41·85	—	76·0	82·0	—	—
2	—	60	70	51·37	—	100·2	72·4	—	—
3	—	63	72	52·33	—	98·9	83·4	—	—
4	—	60	63	42·93	—	97·1	78·3	—	—
5	—	61	66	44·85	—	84·4	74·9	—	—
6	—	62	70	47·70	—	100·9	74·4	—	—
7	—	62	70	47·70	—	88·9	82·2	—	—
8	—	60	66	45·41	—	89·6	73·3	—	—
9	—	60	68	50·44	—	96·7	78·4	—	—
10	—	62	69	51·63	—	101·6	82·1	—	—
11	—	60	66	47·23	—	95·6	83·4	—	—
12	—	60	67	50·56	—	106·4	82·2	—	—
				1st experimental period					
1	Cheddar	67	78	46·41	622·3	90·5	203·1	81·7	100·0
2	Cheshire	75	82	47·29	627·0	104·0	238·9	74·9	99·0
3	Stilton	72	86	55·44	712·4	125·9	264·4	75·4	98·2
4	Cheddar	70	81	49·47	663·3	110·4	207·3	82·3	99·9
5	Cheshire	71	84	52·32	693·9	120·7	235·3	78·5	98·2
6	Stilton	76	92	55·99	719·4	138·2	256·4	76·2	98·0
7	Cheddar	71	80	45·16	605·6	94·6	232·6	75·8	99·1
8	Cheshire	64	69	34·45	656·8	85·1	194·9	72·6	96·6
9	Stilton	70	78	43·47	558·6	98·0	212·7	76·5	98·1
10	Cheddar	73	80	48·44	649·5	123·1	245·1	75·8	95·8
11	Cheshire	68	80	49·49	656·3	109·5	234·5	77·7	98·8
12	Stilton	71	85	55·49	713·1	130·4	257·3	76·8	98·6
				2nd experimental period					
1	Cheshire	89	100	59·78	792·9	132·4	328·3	71·7	98·9
2	Stilton	89	103	59·21	760·8	137·2	320·7	70·0	98·8
3	Cheddar	98	106	52·51	704·2	136·1	305·5	72·0	97·0
4	Cheshire	92	106	64·06	849·6	168·6	296·7	77·1	96·6
5	Stilton	93	111	69·98	899·3	173·5	345·7	73·7	98·3
6	Cheddar	104	117	69·19	927·9	188·4	353·0	72·9	96·9
7	Cheshire	87	99	52·43	695·2	114·6	285·2	73·0	99·2
8	Stilton	77	88	46·64	599·4	104·8	225·1	76·7	98·6
9	Cheddar	89	93	46·22	619·7	117·0	253·0	73·7	96·7
10	Cheshire	92	106	68·34	906·3	197·2	319·9	77·5	93·1
11	Stilton	92	112	74·81	961·4	157·9	357·2	74·6	99·7
12	Cheddar	97	111	66·24	888·4	161·2	346·0	74·0	98·6
				3rd experimental period					
1	Stilton	106	118	64·31	826·4	150·0	404·0	64·7	99·0
2	Cheddar	112	124	64·27	861·8	156·3	405·7	65·2	98·8
3	Cheshire	108	120	58·04	769·6	142·4	416·0	61·4	99·2
4	Stilton	117	127	67·47	867·2	183·5	397·9	66·7	95·5
5	Cheddar	121	130	66·04	885·5	188·9	437·4	64·9	97·0
6	Cheshire	124	137	72·63	963·2	216·9	430·0	66·2	95·7
7	Stilton	108	121	66·07	848·9	158·8	387·7	66·9	98·3
8	Cheddar	92	94	39·69	532·2	102·1	301·7	59·4	96·6
9	Cheshire	93	96	38·63	512·3	105·2	291·1	59·4	95·9
10	Stilton	115	122	67·40	866·2	203·5	438·8	62·8	91·9
11	Cheddar	120	136	78·49	1052·0	200·4	412·8	72·0	96·7
12	Cheshire	117	126	68·46	908·0	179·1	422·2	67·1	97·7
				Low egg-nitrogen period					
1	—	122	124	58·28	—	135·3	113·9	—	—
2	—	127	136	68·27	—	161·9	114·7	—	—
3	—	125	139	64·98	—	161·2	132·7	—	—
4	—	131	144	73·79	—	154·8	129·6	—	—
5	—	137	144	71·36	—	187·8	147·3	—	—
6	—	142	153	76·99	—	193·2	123·8	—	—
7	—	121	124	52·33	—	120·3	107·4	—	—
8	—	99	109	55·43	—	120·3	98·1	—	—
9	—	103	112	54·61	—	123·2	94·6	—	—
10	—	121	131	68·20	—	134·7	149·3	—	—
11	—	140	148	78·00	—	165·8	129·1	—	—
12	—	124	131	62·83	—	150·4	127·0	—	—

Table 6. *Mean biological values and true digestibilities of the proteins of the different cheeses*

Experimental period	Biological value			True digestibility		
	Cheddar	Cheshire	Stilton	Cheddar	Cheshire	Stilton
I	78·9	75·9	76·2	98·7	98·2	98·2
II	73·2	74·8	73·8	97·3	97·1	98·9
III	65·4	63·5	65·3	97·3	97·1	96·2
Mean	72·5	71·4	71·8	97·8	97·4	97·8

Table 7. *Statistical analysis of the data in Table* 6

Difference between tested cheeses	Biological value			True digestibility		
	Cheddar-Cheshire	Cheddar-Stilton	Cheshire-Stilton	Cheddar-Cheshire	Cheddar-Stilton	Cheshire-Stilton
Difference	+1·1	+0·7	−0·4	+0·4	0·0	−0·4
S.E.M.	±2·78	±3·04	±2·97	±0·49	±0·65	±0·38
P*	1 : 1	1 : 1	1 : 1	1 : 2	1 : 1	1 : 2
	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

* P = probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population. N.S. = not significant.

(*b*) *The growth-promoting value of the cheeses*

In order to find out whether the processes of ripening had produced any deep-seated changes in the nutritive values of the cheeses an attempt was made to compare these values with that of milk. For this purpose the cheeses were blended with the other product of cheese-making, whey. For practical reasons dried milk and dried whey were used. This procedure is admittedly approximate, as any changes suffered by whey during commercial drying would be superimposed on those normally undergone by the cheese, though this would doubtless be counterbalanced to a certain extent by the effects of drying on milk itself. Ideally, the milk, cheese and whey should have been derived from the same original bulk of liquid milk. Cheese and whey could then be blended in proportions very similar to those in which they were obtained from the milk. For the present purpose it was thought more important to blend to a fat content close to that of the sample of dried milk available. The milk and whey were high-quality spray-dried products. A salt mixture was added to all diets which supplied 15 mg. Fe, 1·5 mg. Cu and 1·5 mg. Mn per 100 g. diet. The composition and analysis of the diets is given in Table 8.

Table 8. *Composition and analysis of the diets used for determining the growth-promoting value of dried milk or of a combination of dried cheese and dried whey*

Component	% of diet			
Cheddar cheese (dried)	58·97	—	—	—
Cheshire cheese (dried)	—	59·56	—	—
Stilton cheese (dried)	—	—	52·82	—
Full-cream spray-dried milk	—	—	—	99·11
Spray-dried whey	40·14	39·55	46·29	—
Salts*	0·89	0·89	0·89	0·89
N	4·288	4·292	3·919	4·098
Fat	29·8	29·8	29·8	28·7
Moisture	9·48	10·02	10·36	4·62
Ca	0·918	0·906	0·386	0·916
P	0·729	0·735	0·537	0·729
Ca/P	1·26	1·23	0·70	1·26

* Iron citrate 88·92%, cupric sulphate ($CuSO_4.5H_2O$) 6·52%, manganese sulphate (anhydrous), 4·56%.

Nine groups of four newly weaned litter-mate male rats, weighing 40–54 g., were allocated by lot to the four diets. The animals were kept in individual cages and their food intakes recorded. The diets were fed mixed with distilled water, but tap water was given for drinking. The experiment lasted 6 weeks. The animals were then killed and their femora and humeri removed for determination of ash content (Kon & Henry (36)).

The results are shown in Fig. 3 and Tables 9 and 10. It will be seen from these that the best gains were made by the animals receiving the milk diet and the poorest by those receiving the Cheshire cheese diet, but these differences lack statistical significance. They are due to slight variations in food intake since the gain in weight per g. diet intake was almost identical for all four groups of animals.

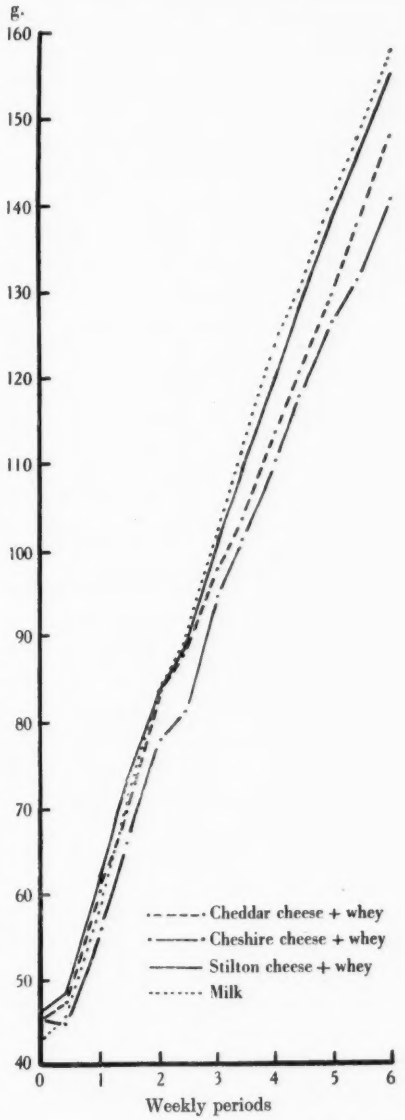

Fig. 3. Growth of rats receiving the milk or cheese-whey diets.

It has been shown by Mattick (37) that Stilton cheese contains much less calcium than other varieties. In fact the diet with Stilton cheese had less than one-half of the calcium present in the other two diets. The percentage of calcium was nevertheless appreciably higher, especially when the contribution of calcium from the tap water is considered, than that of diet B of Sherman & Macleod (38), which contains 0·33%, and at one time was taken as supplying 'normal' quantities of calcium. Nevertheless, it will be seen from Table 10 that the ash content of the bones of rats receiving the Stilton cheese diet was slightly, but significantly, lower than that of bones of other groups of rats. This is in agreement with the observations of Sherman & Booher (39) and of Lanford & Sherman (40) that in the early stages of growth in the rat increases in the calcium content of the diet above that of diet B are accompanied by increased mineralization of the bones. Apart from confirming the obvious fact that Stilton cheese is, relatively speaking, very low in calcium and therefore nutritionally inferior to the hard varieties, these experiments have not disclosed any marked deterioration of the nutrients present in that part of milk which is converted into cheese.

Table 9. *Growth performance of rats receiving milk or cheese-whey diets*

Litter no.	Weight of rat g. Initial	Final	g. diet intake (dry basis)	g. gain in weight/g. diet intake	% bone ash	Weight of rat g. Initial	Final	g. diet intake (dry basis)	g. gain in weight/g. diet intake	% bone ash
	Cheddar cheese diet					Cheshire cheese diet				
1	50	138	269·9	0·33	60·7	46	144	272·0	0·36	60·8
2	46	148	292·4	0·35	61·4	46	135	267·7	0·33	61·8
3	43	171	345·0	0·37	62·6	42	148	315·0	0·34	60·9
4	43	157	339·9	0·34	61·8	44	159	323·0	0·36	63·3
5	50	155	304·8	0·34	61·6	54	142	292·8	0·30	61·7
6	46	95	213·0	0·23	59·5	47	89	203·2	0·21	58·7
7	43	130	324·9	0·27	61·0	48	152	299·0	0·35	60·5
8	40	148	333·9	0·32	61·4	42	161	315·4	0·38	62·8
9	46	182	350·1	0·39	60·6	45	140	299·8	0·32	60·9
Mean	45·2	147·1	308·2	0·33	61·2	46·0	141·1	287·5	0·33	61·3
	Stilton cheese diet					Milk diet				
1	50	144	269·6	0·35	58·2	42	165	328·4	0·37	60·6
2	47	141	279·9	0·34	58·2	48	151	316·4	0·33	60·6
3	42	131	296·2	0·30	60·9	40	148	322·8	0·33	61·5
4	42	169	335·3	0·38	58·8	40	166	337·0	0·37	60·9
5	54	177	323·6	0·38	60·1	47	196	392·4	0·38	61·3
6	48	141	302·6	0·31	57·3	46	135	312·1	0·29	58·6
7	46	167	348·5	0·35	59·8	40	167	356·0	0·36	61·2
8	46	168	342·6	0·28	59·3	44	126	297·5	0·28	60·9
9	46	156	294·9	0·37	58·8	46	163	369·8	0·32	59·8
Mean	46·8	154·9	310·4	0·34	59·0	43·7	157·4	336·9	0·34	60·6

Table 10. *Statistical analysis of data in Table* 9

Difference tested between diets	Gain in weight (g.) Numerical difference	S.E.M.	P*	% bone ash Numerical difference	S.E.M.	P*	
Cheddar-Cheshire	+ 6·8	±6·2	1 : 3 N.S.	−0·1	±0·34	1 : 1	N.S.
Cheddar-Stilton	− 6·2	±8·9	1 : 2 N.S.	+2·2	±0·39	1 : 1667	S.
Cheddar-Milk	−11·8	±9·6	1 : 4 N.S.	+0·6	±0·15	1 : 250	S.
Cheshire-Stilton	−13·0	±9·6	1 : 5 N.S.	+2·3	±0·49	1 : 625	S.
Cheshire-Milk	−18·6	±9·2	1 : 13 N.S.	+0·7	±0·36	1 : 9	N.S.
Milk-Stilton	+ 5·6	±6·8	1 : 2 N.S.	+1·6	±0·56	1 : 47	S.

* See footnote, Table 7. S. = significant; N.S. = not significant.

Summary

1. Cheshire, Cheddar and Stilton cheeses were made on one occasion from winter, and on another from summer, milk.

2. The distribution of vitamin A and carotenoids, vitamin B_1, riboflavin and vitamin C between milk, whey and curd was measured during cheese-making.

3. The fate of these vitamins during ripening was determined.

4. Some 80% of vitamin A and carotene, 15% of vitamin B_1 and 25% of riboflavin originally present in milk were recovered in the green cheese and persisted throughout ripening.

5. Vitamin C disappeared progressively during cheese-making and was absent from the cheeses.

6. Vitamin B_1 and especially riboflavin were recovered in cheese in larger proportions than would be expected from the partition of water between curd and whey, indicating that they were partly present in milk in combination with the coagulable solids.

7. The concentrations of the various vitamins in milk and in the ripe cheeses were respectively for the winter experiment: vitamin A 15 i.u./g. fat and 13 i.u./g. fat; carotenoids 1·8 μg./g. fat and 2·0 μg./g. fat; vitamin B_1 33·5 μg./100 g. and 53 (Cheddar), 44 (Cheshire) and 80 (Stilton) μg./100 g.; riboflavin 84 μg./100 g. and 300 (Cheddar), 260 (Cheshire) and 240 (Stilton) μg./100 g. For the summer experiment the values were: vitamin A 28 i.u./g. fat and 25 i.u./g. fat; carotenoids 10 μg./g. fat and 9–10 μg./g. fat; vitamin B_1 42 μg./100 g. and 55 (Cheddar), 48 (Cheshire) and 75 (Stilton) μg./100 g.; riboflavin 128 μg./100 g. and 330 (Cheddar), 330 (Cheshire) and 360 (Stilton) μg./100 g.

8. There was no difference between the three types of cheese made in winter in biological value or true digestibility of the proteins for which values of 72 and 98 respectively were obtained at an 8% level of protein intake by the method of Mitchell (26, 27).

9. Diets were blended from dried Cheshire, Cheddar or Stilton cheese and from dried whey to approximate the composition of a sample of dried milk. These diets, when supplemented with iron, copper and manganese and given as an exclusive diet, produced growth in rats as good as that obtained with the mineralized milk itself.

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326. THE EFFECT OF TEMPERATURE OF PRE-HEATING, OF CLARIFICATION AND OF BACTERIOLOGICAL QUALITY OF THE RAW MILK ON THE KEEPING PROPERTIES OF WHOLE-MILK POWDER DRIED BY THE KESTNER SPRAY PROCESS

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(With 10 Figures)

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INTRODUCTION

Objects of experiment

Before the present war large quantities of spray-dried full-cream milk powder were prepared in America, but manufacture in this country was on a more limited scale. One of the disadvantages of such powder is its comparatively short keeping quality due to the development of the tallowy off-flavours accompanying atmospheric oxidation of the fat.

The conditions imposed by the war made it necessary to explore all practicable means of extending the keeping quality of milk powder, and an account has already been published of the success attending packing under inert gas (see Part I, ref. 1). This method, however, requires special factory equipment and great care in the manufacture, sealing and testing of the cans to ensure absolute gas tightness.

Simpler methods of extending the keeping quality of spray-dried powder to 12 months or more are clearly desirable.

The present experiments were undertaken to determine the effects of variations in the methods of preparation of the powder, of plant cleanliness and sterility and of the handling of the raw milk upon the chemical, physical, nutritional and bacteriological properties of the powder and upon its keeping quality.

The work formed part of a joint programme of the Agricultural Research Council and the Food Investigation Board of the Department of Scientific and Industrial Research. Dr A. T. R. Mattick, of the National Institute for Research in Dairying, was responsible for the procedures adopted in the factory, with the co-operation of Miss E. R. Hiscox, National Institute for Research in Dairying, and Mr E. L. Crossley, of Messrs Aplin and Barrett Ltd., who made bacteriological and other determinations at the Frome and Yeovil, Somerset, factories of this company. Dr C. H. Lea, Low Temperature Research Station, Cambridge, and Mr J. D. Findlay and Dr J. A. B. Smith, of the Hannah Dairy Research

Institute, determined separately the keeping qualities of the milk powders prepared at each stage of the experiment. Dr S. K. Kon and Mr S. Y. Thompson, National Institute for Research in Dairying, determined the stability of the vitamins in the various powders and made observations on flavour stability of the reconstituted milks. Mr J. W. Egdell, University of Bristol, tested and selected the milk supplies during the experimental period.

At the suggestion of the Ministry of Food, Messrs Aplin and Barrett readily agreed to place at the experimenters' disposal their milk-drying plant at Staplemead, Frome. The experiments were carried out on a commercial scale, and with the least possible disturbance of factory routine. Enthusiastic co-operation was given by the foremen and working staff, and the experimenters' thanks are due in particular to Mr S. A. Wilcox, manager of the factory, for his assistance in ensuring the smooth introduction of such alterations to the ordinary routine as were considered necessary.

There were special advantages in the selection of this factory for the experiment, since Crossley & Johnson(1) had, over a period of years, succeeded in raising the general standard of plant operation to a high level, and considerable knowledge of the effect of various factors on the chemical, physical and bacteriological qualities of the dried milk had been acquired.

The experiments were made in July and August 1942. The main results were communicated by the Ministry of Food to managers of dried milk factories in England, and were applied in the 1943 season. The full results of the work are now published by permission of the Agricultural Research Council and the Department of Scientific and Industrial Research, and with the concurrence of the Ministry of Food.

Type of plant

The plant used was of modern design and constructed mainly in stainless steel. Except that a few small exposed copper surfaces (on ancillary plant) were removed or retinned, no plant alterations were necessary. The processes consisted essentially of pre-heating the milk in tubular heaters, and then concentrating to about 42% solids by means of a triple circulation climbing film evaporator; the concentrated milk was dried by spraying into a hot-air chamber in the form of a fine mist produced by a rotating disk atomizer. The very large evaporating and drying plants were of the Kestner type and were housed in a four-storey building.

The general lay-out of the plant is illustrated diagrammatically in Fig. 1 (not to scale). After reception and examination on the receiving platform, the raw milk was tipped into a weighing tank (*A*), thence pumped (*B*) through a clarifier (*C*) to a balance tank (*D*). In those trials when the clarifier was not used, the milk passed directly to the balance tank, which fed a plate type (A.P.V.) cooler (*F*). By means of a pump (*E*), the milk was forced through the cooler (*F*) and the cooled raw milk passed to a series of large storage tanks (*G*) fitted with paddle stirrers. (Some only of the storage tanks are shown in Fig. 1.) The bulked raw milk then flowed by gravity over open corrugated heaters (*H*) which raised its temperature to about 110–115° F. Two such heaters, constructed of tinned copper and heated internally by steam, were employed in rotation and delivered the warmed milk by gravity into a small aluminium feed tank (*I*). The milk was then raised to the final pre-heating temperature of either 165 or 190° F. by pumping (*J*) through a Scott type tubular heater (*K*), wherein the milk flowed through a series of

steam-heated tubes. (Only one tubular heater is shown in Fig. 1, but two such heaters were employed in rotation to facilitate cleaning.) The time of passage through the tubular heater was about 20 sec. and the process was therefore a form of continuous high temperature-short time pasteurization. The hot milk was delivered to a balance tank (*L*) feeding the evaporator, in effect introducing an additional holding period of 3–5 min. Continuous and very rapid concentration to about 42% solids at a temperature of 115–120° F. was effected by passage through a large climbing film evaporator

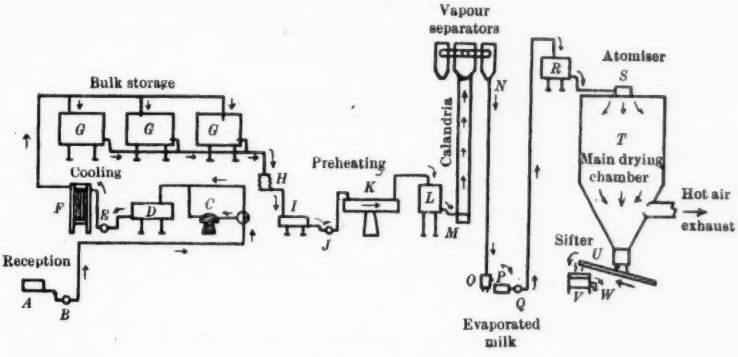

Fig. 1. General plant lay-out (not to scale).

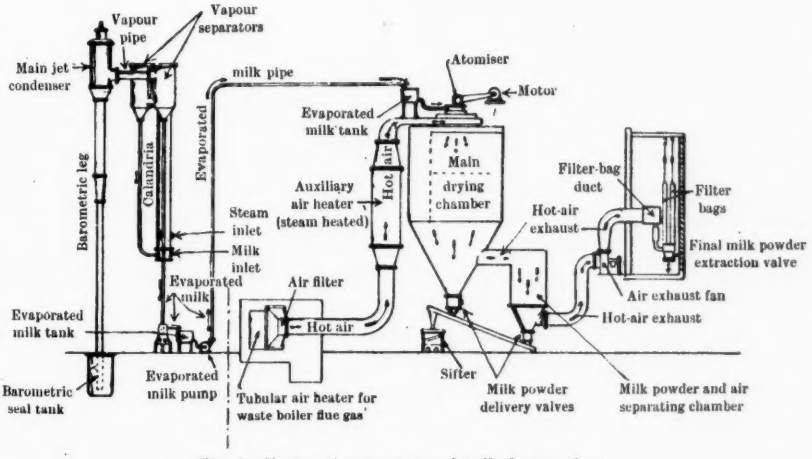

Fig. 2. Kestner 'spray process' milk drying plant.

(*M* to *N*) calandria, details of which are not shown in Fig. 1. The concentrated milk flowed through a small cylindrical testing tank (*O*), wherein the specific gravity was frequently observed by hydrometer, to a small balance tank (*P*). From this point the concentrated milk was raised by a pump (*Q*) almost to the top of the building through a long pipe-line to two atomizer feed-tanks (*R*), one of which is figured. From the atomizer feed-tanks the concentrated milk passed via the atomizer (*S*) to the drying chamber (*T*). (Details of the drying plant are shown in Fig. 2.) The bulk of the powder was discharged from the main chamber and a cyclone separator (shown in Fig. 2) into

a conveyor (*U*) which delivered it to a sifter (*V*). The final sifted powder was discharged from sifter outlets (*W*) into suitable containers. The small proportion of powder recovered by means of filter bags from the exit air (Fig. 2) was not examined during this experiment, since this powder is normally of inferior quality(1).

The Scott heater tubes, calandria tubes, balance tanks, storage tanks, and pipe-lines were constructed entirely of stainless steel, whilst the drying chamber was constructed of tinned steel.

Schedule of experiments

Experimental runs extending over the period 27 July to 22 August 1942 (a very hot spell) were carried out according to the plan shown in Table 2.

Sampling in the factory

The following samples for examination were taken during each experimental run:

Raw milk from the bulk tank (*G*).

Pre-heated (pasteurized) milk at the inlet to the balance tank (*L*) and occasionally from the balance tank.

Concentrated milk: (1) at the outlet from the evaporator (*O*), (2) at the inlet to the atomizer feed-tank (*R*).

Dried milk powder from the sifter (*V*) outlet and occasionally from the conveyor (*U*).

Sampling, requiring two persons, was carefully timed so that each sample represented one small bulk of milk at various stages of its progress through the plant. Thus, if the time of sampling the raw milk is taken as 0 min.:

Pre-heated milk was sampled at 3 min.
Concentrated (1) was sampled at 18 min.
Concentrated (2) was sampled at 19 min.
Dried milk powder was sampled at 21 min.

The samples were collected in sterile bottles (where necessary with the aid of sterile dippers), the hot samples quickly cooled with running water and the powder samples tightly sealed. The milk samples were plated immediately, but the powder was tested up to 26 hr. later in the laboratory at Yeovil.

Before the test period of each run a bulk of milk (not less than 500 gal.) was isolated in a tank fitted with an automatic stirrer. This was sufficient to feed the plant for at least an hour, and the bulk sample of powder required for the keeping quality tests was therefore produced from the same bulk of milk that was tested at the beginning of the run. Thus the results of the tests on the bulk powder could be related to the findings of the tests on the samples collected from various points in the plant.

The bulk sample of powder was collected in a clean dry metal tub placed at the outlet pipe of the sifter. About $\frac{1}{2}$ hr. was required to collect the necessary quantity, and during this time the sample was exposed to air contamination. In the earlier trials there was considerable risk of small quantities of loose powder being thrown on the sifter and no effort was made to avoid draughts from open doors. But when the operatives appreciated more fully the aims of the experiment, precautions were taken to reduce air and other contamination to a minimum. On five occasions during the experimental period parallel test samples of powder were taken direct from the main cone, i.e. before exposure to air contamination on the sifter and from the sifter outlet. There was close agreement in the plate counts of the powder from the two sampling points indicating the absence

of serious contamination on the sifter at this stage of the experiment. Crossley & Johnson(1) had previously obtained similar results from twenty-seven similar comparisons. Samples taken direct from the sifter into sterile bottles at intervals during one run showed no significant differences in plate count, indicating that, in the absence of air contamination, the organisms present were fairly evenly distributed throughout the bulk of powder (Table 1). This was an important point, since it was intended to make bacteriological tests of the powder after storage. A further indication of the consistency of the plate count of the powder was provided by counts, made by Dr C. Higginbottom at the Hannah Institute, of powder collected by an independent worker at intervals during the runs on 4 days during this period. Later, serial platings of tins of powder during the storage period showed a difference, significant though small, between ten tins of 22-2-G and five tins of 13-2-G after about 12 months' storage.

The powder was carefully packed by hand into sterilized dry 1 lb. tins free from all visible traces of exposed iron or faulty tinning, and sealed by means of a small machine. A number of tins, gas-packed by the ordinary factory process, was reserved for bacteriological and chemical tests by Mr E. L. Crossley and by the workers at the National Institute for Research in Dairying. The remainder (air-packed) were sent to the Low

Table 1. *Samples taken from sifter at close intervals, 23 August 1942*

Time	Moisture	Solubility*	Acidity % lactic acid	Plate count 37° C.†
12.0	—	2·0	0·90	310
12.20	4·1	2·7	0·95	—
12.25	4·2	2·1	0·86	330
12.40	3·8	2·0	0·95	—
12.50	3·9	2·0	0·90	430

* Determined by the method laid down by the American Dry Milk Institute.

† Coli, anaerobes and moulds absent from all samples.

Temperature Research Station, Cambridge, the Hannah Institute, Aplin and Barrett, Ltd. and the National Institute for Research in Dairying in the numbers requested by these centres.

An attempt was made to be consistent in tightness of packing, but without weighing each tin it was impossible with hand packing to avoid some variation in the weight of the samples. Speed in packing was considered essential, and since on some occasions eighty-two tins were required from one bulk sample, weighing was omitted. Samples 5–1 and onwards were rammed tightly into the tins (by means of a sterilized wooden ram-rod) to simulate as nearly as possible the commercial packing in the Frome factory, and each tin contained approximately $1\frac{1}{4}$ lb. The tins for gas packing were less tightly packed.

The samples were accumulated in a cool cellar at Messrs Aplin and Barrett's factory at Yeovil, whence they were distributed to the various centres on 20 September 1942.

For the bacteriological tests and vitamin assays at the N.I.R.D. the tins were stored at room temperature and used as required.

During the experimental period (trials 5–8) special measures were taken to improve the freshness and the bacteriological quality of the raw milk used for drying. The milk was collected from the farms twice daily and transported direct to the factory so that all supplies could be dried within a few hours of milking. Distant supplies were diverted to other centres. On arrival at the factory the supplies from every producer were tested

by the 10 min. resazurin test, and only those giving a disk reading of 6 or 5½ were accepted for drying.

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PART I. KEEPING PROPERTIES: FACTORS INFLUENCING STABILITY

By C. H. Lea, J. D. Findlay and J. A. B. Smith

As long ago as 1926, Holm, Greenbank & Deysher (2) observed that butter fat prepared from milk less than 12 hr. old was less susceptible to oxidation than fat separated from milk 1 day old. They also claimed that removal of 'separator slime' containing cellular material from the milk by use of a centrifugal clarifier improved the keeping properties of the powder. Finally, they found that pre-heating of the milk for 30 min. at 181° F. before drying gave a powder of better keeping properties than when 145, 163 or 200° F. was used. Very few details were given and it is difficult to judge the order of magnitude of the effects that were obtained.

Few further data appear to have been published on the effect of freshness* and bacteriological quality of the milk, or on the use of the clarifier, but a considerable volume of indirect evidence on the stabilizing effect of high pre-heating temperatures in various milk products has accumulated.

Kende (3) found that the oily (oxidative) taint which develops in some milks, particularly at low storage temperatures, could be prevented by heating the milk at 185° F. for 5 min., and that reducing substances were formed in milk heated to 176–185° F. Subsequently, other observers have stated that heating to 170° F. for 10 min. is sufficient to prevent the development of oxidized flavours in susceptible milks.

High-temperature treatment of cream has been found advantageous in producing butter resistant to the development of oxidative taints in cold storage. Scheib, Stark & Guthrie (4) found that exposure to 165° F. for 30 min. was necessary, 15 min. being insufficient. Cream itself is much more resistant to spoilage when flash pasteurized at 170–190° F. than when heated at 150° F. for as long as 30 min. (5). Trout (6) recommends pre-heating cream intended for cold storage at not less than 165° F. for 15 min. and preferably at 185° F. for 5 min. A cooked flavour is produced by this treatment, but it is not considered objectionable.

Recently, Hollender & Tracy (7) have found that dried milk prepared in the laboratory on a very small vacuum roller drier showed better keeping properties when the milk was pre-heated at 170° F. for 30 min. than when 150 or 190° F. was used. Jack & Henderson (8), by pre-heating at 175° F. for 15 min., produced an atmospheric roller powder which kept in good condition for over 2 years, while the control pasteurized at 142° F. for 30 min. kept only for 5 months. This treatment produced a more markedly cooked flavour in the dried milk, but not in such degree as to be objectionable. A spray-dried powder pre-heated at 220° F. for 10 sec. was said to have kept for over 2 years, while the control powder kept for only 3 months. No details of the preparation or storage of these powders were given.

* One of the authors has observed that dried milk produced by the freeze-drying process had much better keeping properties when made from fresh milk than when a staler, but still good, portion of the same sample of milk was used. The milk was pasteurized at 145° F. for 30 min.

THE EXPERIMENTS

In the present work attempts have been made to ascertain the effect on the keeping properties of the powder of

(*a*) improving the bacteriological quality of the raw milk by eliminating unsatisfactory sources of supply and by more frequent collection, etc., with the object of minimizing the proliferation of micro-organisms and the production in the raw milk of oxidizing enzymes, acids, etc., which might accelerate spoilage of the dried powder;

(*b*) centrifugal clarification of the milk, thereby removing in the separator slime cellular matter which might supply enzymes to the milk;

(*c*) reduction to the lowest practicable level of contamination by metals, such as copper and iron known to have harmful effects on the keeping properties of the powder;

(*d*) more careful cleaning and sterilizing of the plant in order to minimize the growth of micro-organisms in the milk during processing;

(*e*) pre-heating the milk at a higher temperature than normal, with the object of obtaining greater destruction of bacteria and of harmful enzymes, and of producing

Table 2. *Schedule of experiments*

Trial no.	Inclusive dates (1942)	Milk supply	Collection daily	Clarifier	Pre-heating temperature* °F.
1	27–29 July	Ordinary	Once	Not used	165
2	30 July–1 Aug.	,,	,,	Not used	190
3	3–5 Aug.	,,	,,	Used	190
4	6–8 Aug.	,,	,,	Used	165
1A†	9 Aug.	,,	,,	Not used	165
5	10–12 Aug.	Selected	Twice	Used	190
6	13–15 Aug.	,,	,,	Not used	165
7	17–18 Aug.	,,	,,	Not used	190
8	19–20 Aug.	,,	,,	Used	165
5A‡	21–22 Aug.	,,	,,	Used	190

N.B. For the coding of the individual samples constituting each trial see Table 13.

* Duration about 20 sec., followed by 3–5 min. at a slightly lower temperature, in the holding tank.

† Procedure as 27–29 July, but cleaner plant. ‡ Procedure as 10–12 August.

from the milk protein sulphydryl-containing substances likely to function as anti-oxidants in the dried powder.

The keeping properties of the various powders were investigated both at the Hannah Institute and at the Low Temperature Research Station, but since different procedures were adopted at the two institutes the results are summarized separately below.

THE EXPERIMENTAL MILKS

The procedures adopted at the factory and the key numbers by which the samples are indicated are given in Table 2. A number of 1 lb. tins of powder collected during every morning and afternoon of the run served as material for the storage tests, which were carried out at 47, 37 and 15° C. or room temperature. Since nearly a month elapsed between preparation of the first and last samples of the series the powders were placed at 47 and at 37° C., at such intervals that all would be roughly of the same storage age when tasting tests began. For this purpose 1 day at 15° C. was considered equivalent to $\frac{1}{4}$ day at 37° C. or $\frac{1}{8}$ day at 47° C. Any error involved in making this assumption would be negligible by comparison with the duration of the storage experiments.

Moisture and solubility. The moisture content and solubility of the various powders are given in Table 3. Solubility was determined by the rapid method of Howat, Smith, Waite & Wright (9), a correction being made to allow for the hydration of lactose during the determination.

It is obvious from Table 3 that the different treatments to which the liquid milk was subjected had no appreciable effect on the solubility of the powder, and that the solubility of all samples was typical of many normal spray-dried powders. The moisture contents of all the powders were sufficiently low to ensure that no loss of solubility should occur during any reasonable period of storage (1).

Flavour. Examination of the fresh powders divided them into two easily distinguishable groups, according to the temperature at which the milk had been pre-heated.

The powders of the low-temperature group were very palatable and tasted cool and fresh, but had that characteristic odour and flavour frequently associated with spray-dried full-cream powder, which is possibly due to the earliest stage of tallowiness. Differences between the individual powders of the group were slight.

Table 3. *The solubility and moisture and copper contents of the various powders**

Trial no.	Sediment at 20° C.	Sediment at 50° C.	Solubility at 20° C.	Solubility at 50° C.	Moisture (%) Hannah Institute samples	Moisture (%) Cambridge samples	Copper (p.p.m.) Hannah Institute samples	Copper (p.p.m.) Cambridge samples
1	0·3	0·05	92	98	1·7	1·8	0·6	0·7
2	0·3	0·05	93	98	1·9	1·9	0·7	0·7
3	0·3	0·05	92	98	1·2	1·6	0·6	0·6
4	0·3	0·05	92	98	1·9	1·9	1·0	1·3
5A	0·3	0·05	93	100	2·2	1·7	0·6	0·6
6	0·3	0·05	93	100	1·5	1·9	0·6	1·3
7	0·3	0·05	92	100	1·8	1·8	0·6	0·8
8	0·3	0·05	94	100	2·2	1·8	0·8	0·7

* As explained on p. 130 the samples stored at the two laboratories were not identical. Moisture contents were determined by air-oven methods. Determination by vacuum oven on the Cambridge samples gave values averaging 0·3% higher.

The powder from the milks pre-heated at the high temperature all possessed a 'boiled milk' or 'cooked' odour and, in rather less degree, taste, but were fresh-tasting and palatable. The boiled taste seemed less marked in 3 and 5 than in 2, 5A and 7.

The opinion of the tasting panels was that the slight 'boiled' odour and flavour was not objectionable. It was of a type frequently associated with milk and was much less strong than in truly boiled or sterilized milk. It was not similar to the 'heated' or 'roller powder-like' taste sometimes present in fresh or stored spray powders, which includes an element of staleness.

Sulphydryl content. The nitroprusside test for sulphydryl compounds was carried out by a method very similar to that described by Josephson & Doan (12). Weigh 1 g. of powder into a test-tube, add 5 ml. water and shake for 1 min. Add 5 g. 'analar' ammonium sulphate and shake again. Cool in ice-water for 2 or 3 min. Add a few drops of 0·88 ammonia, shake well, and then add 5 drops of freshly made 5% sodium nitroprusside. Shake the tube and replace it in the ice-water. If the test is positive a pink colour develops immediately and lasts for 15 or 20 min.

The powders from the milk pre-heated at the high temperature all gave a marked pink colour when submitted to this test, whereas with those from milks pre-heated at the low temperature only a slight brown tinge was observed.

Also satisfactory was a modification of the test for hydrogen sulphide employed by Gould & Sommer(10) in investigating the influence of temperature on the production of cooked flavour in fresh milk. 200 g. of powder in 1 l. of water were heated in a water-bath to 60° C. and nitrogen blown through at the rate of 100 l. per hour, the gas after passing through the milk being forced through a disk of Whatman no. 52 filter paper approximately 2 cm. in diameter moistened with a solution of basic lead acetate. All the powders from the milks pre-heated at the low temperature gave completely negative reactions over a period of 60 min. All the powders from the milks pre-heated at the high temperature showed a positive reaction within 3 min., and after 30 min. the disk was uniformly blackened to an intensity which varied somewhat with the sample, nos. 3 and 5 being palest and nos. 2 and 5A darkest. Alternatively, the volatile sulphur could be collected in alkaline zinc acetate solution and estimated colorimetrically, after conversion to methylene blue, as in the procedure recently described by Townley & Gould(11). All the 'low-temperature' powders gave negative reactions, and all the 'high-temperature' powders strong positive reactions. The powders were nearly 12 months old at the time this test was applied, but had been stored at −20° C. in gas pack for most of the period.

Copper content. Copper was estimated by the method of Sylvester & Lampitt(13) using a Spekker absorptiometer or Zeiss-Pulfrich photometer and CCl_4 instead of amyl alcohol. From the results recorded in Table 3 it will be seen that the copper contents were uniformly low in samples 1, 2, 3, 5A, 7 and 8, slightly higher in 6, and greatest in sample 4, where values of 1·0 and 1·3 p.p.m. were found. Since the copper content of liquid milk has been found to vary between 0·09 and 0·17 p.p.m. with an average of 0·12 p.p.m.(13), these values are probably minimal for dried milk.

Storage tests at Cambridge

Technique. Samples 1 and 1A were similar in flavour and were combined; samples 5 and 5A were appreciably different in this respect and were stored separately. Nine samples of powder were therefore available, each consisting of about forty 1 lb. cans representative of the output from the plant during the particular experimental period. The powder in each batch was thoroughly mixed and packed in A1 (315 ml.) 'open-top' cans, each containing 5 oz. of powder. For each examination a can was inverted, opened, and 2 oz. of the powder removed. Some of the cans were of plain, and some of lacquered tinplate. A number were gas-packed to an oxygen content (after desorption) of 1 or 3%, and some of the former were stored at −20° C. for use as controls in the tasting tests. The gas packing of these samples could not be carried out until 2 months after manufacture. Constant temperature chambers at 47 and 37° C. were used for the accelerated tests, and at 15° C. for normal storage.

The powders were tasted by the panel approximately 30 min. after reconstitution in the ratio of 14 g. of powder to 100 ml. of water at 20° C. Marks were awarded according to the scale: 6 = very good, 5 = good, 4 = fairly good, 3 = fair, 2 = rather poor, 1 = poor, and 0 = very poor. Spaces were provided on the score sheet for the identification and evaluation wherever possible of 'boiled', 'heated', 'tallowy', 'stale' or 'cardboard' flavours. A mark of 6 would be awarded only to a sample approximating very closely in flavour to fresh milk. Samples scoring between 5 and 6 were good, palatable dried milks, but might have a slightly 'boiled', 'heated' or 'incipient tallowy' taste which did not seriously impair palatability. In milks scoring 4, 3 and 2 tallowy or other 'off'

flavours were increasing from slight to marked, and samples scoring 1 or 0 were very tallowy and unpleasant to taste. A score of 0 did not mean that the sample had reached the worst state possible, but simply that it was very bad.

In order to maintain a consistent standard of marking it was found necessary to include in every group of samples to be tasted some which were quite free from tallowiness. The influence of fatigue or increasing toleration could clearly be seen in the higher scores recorded when several tallowy samples were tasted alone, without non-tallowy

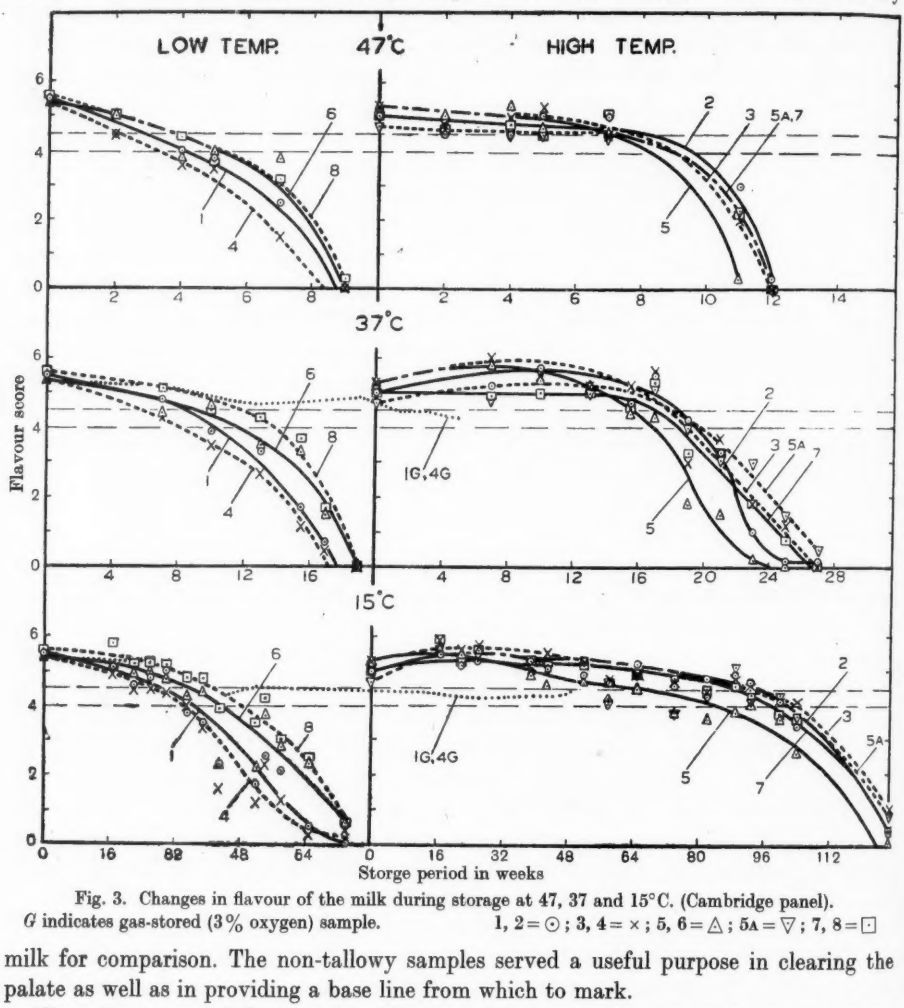

Fig. 3. Changes in flavour of the milk during storage at 47, 37 and 15°C. (Cambridge panel). *G* indicates gas-stored (3% oxygen) sample. 1, 2 = ⊙; 3, 4 = ×; 5, 6 = △; 5A = ▽; 7, 8 = ⊡

milk for comparison. The non-tallowy samples served a useful purpose in clearing the palate as well as in providing a base line from which to mark.

All tasting was carried out under 'blind' conditions, the identity of the samples being unknown to the tasters.

Results. The results of the tasting tests on the samples stored in air at 47, 37 and 15°C. are given in Fig. 3, the 'low' and 'high' temperature milks being plotted separately. The tests at 15°C., which have now been completed, confirm the results for the higher temperatures. Data for samples 1 and 4 stored in nitrogen containing 3% of

oxygen at 37 and 15° C. are also included in Fig. 3. These two samples behaved very similarly, and mean values of the tasting results are plotted. The corresponding powders packed in nitrogen containing 1% of oxygen were examined only after 21½, 24½ and 30 months at 15° C., when they showed palatability scores averaging 0·8 point higher than the 3% samples. Table 4 gives the estimated times for the air-packed samples to deteriorate to flavour scores of 4·5 and 4·0. The latter might be considered the end of the storage life of the milk as a product of reasonably good quality.

Lacquered cans. On a number of occasions samples of the milks which had been stored for various periods at 37° C. in lacquered tinplate open-top' cans* were compared with the corresponding samples in ordinary tinplate containers. In all, fifty-four direct comparisons of plain versus lacquered tinplate were made, thirty-seven being on 'low' temperature and 17 on 'high' temperature samples. No consistent difference was observed between the two types of container, an average score of approximately 2·6 for the whole series being recorded in both cases.

Hollender & Tracy (7) have recently found roller-dried full-cream milk powder to show considerably better keeping quality in lacquered than in plain tinplate containers. The discrepancy between their result and that now reported may be due to the fact that a large proportion of the fat in a roller-dried powder is in a relatively 'free' or easily extractable condition, while the proportion of such fat in the spray-dried powders stored in the present experiments was exceptionally low, as shown by the figures given in Table 5. Other samples of spray-dried powders examined in more recent experiments have shown somewhat higher proportions of readily extractable fat, and significantly better keeping properties in lacquered than in plain tinplate containers. These results will be reported later.

Storage tests at the Hannah Institute

Technique. Storage tests were carried out for all the trials except no. 5. Nos. 1 and 1A were combined. From a large number of 1 lb. samples taken at a given time during each trial, ten 1 lb. cans were selected at random and their contents well mixed. The eight samples so obtained were packed into small glass bottles and plain tinplate cans and stored at 47, 37° C. and room temperature. Some additional cans, both air-packed and nitrogen-packed, were kept at 0° C. as controls.

For the tasting tests samples were reconstituted at 25° C. in eight times their weight of water, labelled in a code unknown to the tasters, and tasted ½–1 hr. later by five people accustomed to such work. Each taster wrote a brief description of the flavours he observed, and a mark was awarded to the sample according to the following scheme: 0 = very good and very like ordinary fresh milk, 1 = fairly good and quite palatable, 2 = slightly but definitely unpalatable owing to the presence of slight 'off' flavours, 3 = unpalatable owing to the presence of pronounced 'off' flavours, 4 and > 4 = very tallowy and most unpalatable. A control powder which had been stored at 0° C. was included in each test. The interpretation of the results is based on the average mark awarded by all five tasters.

Results. The average values for powders stored at 47, 37° C. and room temperature are shown in Fig. 4, in which the results have been plotted in pairs, the important difference between the powders in each pair being that one of them was made from

* Double zinnatine lacquered inside, roller coated.

Table 4. *The keeping quality of the powders as determined by both tasting panels*

Trial no.	Nature of milk dried	Pre-heating temp. °F.	Cambridge results: Weeks to deteriorate to flavour score						Hannah Institute results: Weeks to develop 'off' flavour score					
			4·5 At 47°C.	4·0 At 47°C.	4·5 At 37°C.	4·0 At 37°C.	4·5 At 15°C.	4·0 At 15°C.	1·0 At 47°C.	2·0 At 47°C.	1·0 At 37°C.	2·0 At 37°C.	1·0 At lab. temp.	2·0 At lab. temp.
1	Ordinary unclarified	165	2·7	4·0	8·0	10·0	29	36	3·6	5·7	7·7	11·9	42	61
4	Ordinary clarified	165	2·0	3·3	5·5	8·0	26	35	2·1	3·9	6·4	10·0	38	54
6	Selected unclarified	165	3·5	5·0	9·0	11·4	36	43	7·4	8·7	13·6	18·9	47	70
8	Selected clarified	165	3·5	5·2	11·5	13·8	41	48	6·4	7·9	15·1	19·1	47	66
2	Ordinary unclarified	190	8·3	9·6	18·1	19·6	94	100	10·7	12·3	23·3	26·1	100	115
3	Ordinary clarified	190	7·8	9·0	17·8	19·0	94	104	11·4	13·7	30·0	31·3	105	125
7	Selected unclarified	190	7·5	9·0	17·1	18·5	88	98	11·7	13·1	32·9	35·0	120	133
5A	Selected clarified	190	8·0	9·0	18·0	19·6	92	102	13·6	14·6	32·9	34·6	127	>138
Mean for ordinary milk pre-heated to 165°F. (*a*)			2·4	3·7	6·8	9·0	28	36	2·9	4·8 -	7·1	11·0	40	57
Mean for selected milk pre-heated to 165°F. (*b*)			3·5	5·1	10·3	12·6	39	46	6·9	8·3 -	14·4	18·6	47	68
Mean for ordinary milk pre-heated to 190°F. (*c*)			8·1	9·3	18·0	19·3	94	102	11·1	13·0 -	26·7	28·7	103	120
Mean for selected milk pre-heated to 190°F. (*d*)			7·8	9·0	17·6	19·1	90	100	12·7	13·9 -	32·9	34·8	124	>135
Increase in storage life (expressed as ratios) caused by:														
(A) Selecting the milk														
(1) When pre-heating to 165°F. *b*/*a*			1·5	1·4	1·5	1·4	1·4	1·3	2·4	1·7	2·0	1·7	1·2	1·2
(2) When pre-heating to 190°F. *d*/*c*			1·0	1·0	1·0	1·0	1·0	1·0	1·1	1·1	1·2	1·2	1·2	>1·1
(B) Raising the pre-heating temperature from 165 to 190°F.														
(1) With ordinary milk *c*/*a*			3·4	2·5	2·6	2·1	3·4	2·8	3·8	2·7	3·8	2·6	2·6	2·1
(2) With selected milk *d*/*b*			2·2	1·8	1·7	1·5	2·3	2·2	1·8	1·7	2·3	1·9	2·6	>2·0
Selecting the milk and raising the pre-heating temperature *d*/*a*			3·3	2·4	2·6	2·1	3·2	2·8	4·4	2·9	4·6	3·2	3·1	>2·4

Table 5. *Availability of the fat in milk powders*

Type of powder	% fat extracted by light petroleum: Shaking for 5 min. at lab. temp.	Extraction with boiling solvent for 5 hr. at <40°C.	Extraction with boiling solvent for 5 hr. at 40–60°C.
Roller-dried: Sample 1	22·9	23·1	23·4
Sample 2	—	—	24·2
Spray-dried: Low temperature no. 6	0·6	0·6	0·7
High temperature no. 2	0·6	0·6	0·4

milk pre-heated to 165° F. and the other from milk pre-heated to 190° F. The great effect of high pre-heating temperature in increasing the keeping quality of the powder is thus clearly demonstrated.

When the curves in these diagrams pass beyond the tasting value 1·0, slight 'off' flavours were becoming perceptible to the highly critical palates of the tasting panel. When the curves pass beyond 2·0 the 'off' flavours were reaching an intensity at which they would be observed by the average consumer. The relative keeping qualities of the different samples are therefore best seen from the values in Table 4, in which the number of weeks required for each sample to reach tasting values 1·0 and 2·0 are recorded. Storage at room temperature has not yet progressed sufficiently far for 2·0 to be passed by the most stable powder no. 5A.

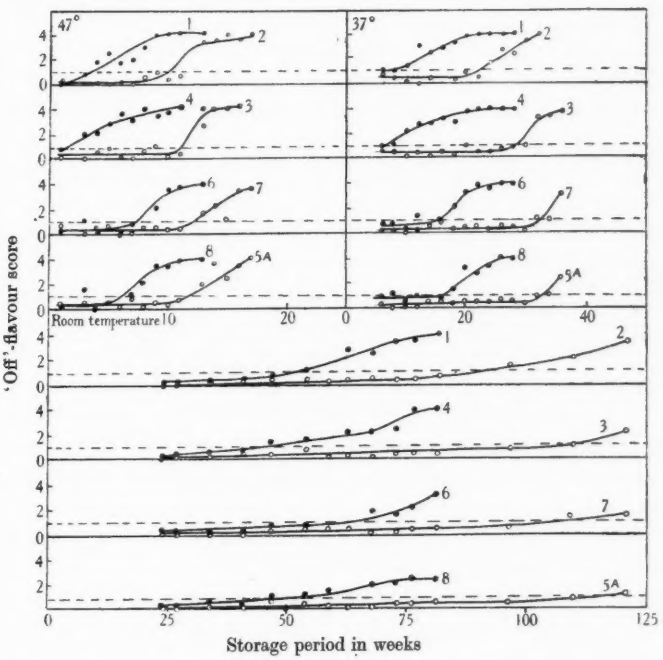

Fig. 4. Changes in flavour of the milk during storage at 47, 37° C. and room temperature (Hannah Institute Panel).

Discussion

The results summarized in Table 4 show that in most of the trials the storage life of the powder as estimated at the Hannah Institute was considerably longer than that estimated at Cambridge. While part of this discrepancy may have been due to a difference in the relative importance attached to 'off' flavours of different types, it nevertheless seems likely that other factors, such as the method of sampling or the conditions of storage of the powders, were responsible for the greater part of the observed difference. That this difference in rate of deterioration was real was shown by determination of peroxide values on the most and least stable Hannah Institute powders (nos. 5A and 4),

which, after 21½ months at room temperature had reached only 5·6 and 19·4 m.equiv./kg. of fat respectively (iodimetric method), as compared with 10·8 and 30·0 for the corresponding Cambridge powders.

Sampling. For the Cambridge tests forty 1 lb. samples were taken at intervals throughout the *whole* period of each trial, and from these forty cans a composite sample was prepared. This was packed into smaller cans for storage. For the Hannah Institute tests, however, the various 1 lb. samples received for storage were taken mainly at only one time during each trial. The composite samples prepared for storage at the two laboratories for any one trial were, therefore, not identical. One represented the powder from a very large bulk of liquid milk dried over a period of days, whereas the other represented one batch of liquid milk dried in the morning or afternoon of one day only.

Storage. Milk powder is a system in which the rate of oxidation of the fat or phosphatide is very susceptible to factors such as access of oxygen and moisture and to the catalytic influence of traces of pro- or antioxidants and of contact with surfaces.* In the present work the containers used at the two laboratories were dissimilar. A further point of difference was that the cans at Cambridge were closed in such a manner as to be not quite airtight, though for all practical purposes they were moisture-proof. Oxygen absorbed by the powder would, therefore, be replaced from the atmosphere. The cans used at the Hannah Institute were closed in the normal manner, and the bottles had waxed-in stoppers. Both were probably gas-tight, so that slowly falling oxygen pressure might have resulted in some extension of the storage life of the powder. Whatever the reason for the absolute difference in storage life observed at the two laboratories, it will be seen from Table 4 and from the following summary that there was nevertheless good agreement in regard to the effect on the keeping properties of the powder of the various factors in handling and processing of the milk which were under investigation.

Effect of pre-heating temperature. All the results show that the most important of the various factors studied was the pre-heating temperature, the powders prepared from milk pre-heated at the higher temperature remaining in good condition, on the average, for two or three times as long as did the powders prepared from milk pre-heated at the lower temperature, the effect of the higher pre-heating temperature being to extend the induction period before rapid deterioration began.

This improvement may have been due to (*a*) more efficient destruction of oxidizing enzymes originally present or produced in the milk by the growth of micro-organisms, or (*b*) the production in the milk of sulphydryl compounds by the action of heat on the proteins, particularly the lactalbumin and the protein associated with the fat globules. The fact that the high pre-heating temperature produced a greater improvement in the keeping properties of powder prepared from the poor than from the good milk supply supports hypothesis (*a*), while the detection of sulphydryl compounds only in the milk pre-heated at the high temperature, and the known antioxidant activity of substances of this type support hypothesis (*b*). Both mechanisms, in fact, are probably involved.

Effect of selection of the milk. Next in importance to the pre-heating temperature, though considerably smaller in its effect, was selection of the milk. All the data agree in

* Preliminary work with butter fat and with milk powder has shown that the type of container used in accelerated storage tests can have a very marked influence on the results. An account of this work will be published later.

indicating that improvement in the quality of the milk supply improved the keeping properties of the powder when the low pre-heating temperature was used. Under the conditions of storage and testing at Cambridge, the average increase in storage life was 40 or 50% at all three storage temperatures. At the Hannah Institute, the average storage life was almost doubled at 47 and 37° C., but it appeared to be increased by only 20% at room temperature. When the milk was submitted to the high pre-heating temperature, the advantage of the improved milk supply became much less marked, no significant change in storage life being detected by the Cambridge panel or in the clarified milk by the Hannah Institute panel, though a small increase (*c.* 20%) was observed with the unclarified milk in the Hannah Institute experiment. Presumably the harmful effect of the growth of micro-organisms in the ordinary milk supply was largely neutralized by the higher pre-heating temperature.

Effect of clarification. With ordinary milk and a pre-heating temperature of 165° F. clarification appeared to reduce the storage life of the powders by about 25% at 47 and 37° C. and by about 10% at 15° C. Since, however, the copper content of the powder made from clarified milk (no. 4) was higher than that of the powder made from unclarified milk (no. 1), it is possible that the shorter storage life of no. 4 may have been due to this cause, rather than to clarification *per se*. The inadequate heat treatment of this powder, as shown by plate counts and phosphatase tests (pp. 140 and 146) may also have been a contributory factor. Results for ordinary milk pre-heated to 190° F. and for selected milk pre-heated to 165 or 190° F. were somewhat variable, individual estimates under the various storage conditions ranging from a reduction in keeping properties of 14% to an increase of 29%, with an average of about 5% in favour of the clarified samples. Some of these differences too may have been due to chance variations in copper content, as in the case of Cambridge samples nos. 6 and 8. It is concluded that clarification in these experiments had very little effect on the keeping properties of the powder.

Keeping properties at the various storage temperatures. Accelerated storage tests were carried out at 47 and at 37° C., the higher temperature being used to give the earliest possible indication of the results. Some powders develop serious heated or caramelized flavours at 47° C., and for general use 37° C. is usually preferred. In the present experiment the powders were of relatively low moisture content and withstood 47° C. fairly well.

Comparison of the results at the two temperatures shows that the powders, on the average, kept for about 2–3 times as long at 37 as at 47° C. (Table 7). If a minimum value of 2·0 per 10° C. be assumed for the range 37–15° C., the storage life at 15° C., of the powder from milks pre-heated at the high temperature should be at least 18 months to 2 years as estimated from the Cambridge data and 2–3 years as estimated from the Hannah Institute data. At the time of writing this report the powders have been stored at 15° C. and at room temperature for over $2\frac{1}{2}$ years, and the predictions of the accelerated tests have, in general, been confirmed (Figs. 3 and 4, Table 4).

Chemical data. Very few chemical tests were made on the powders stored in these experiments, but determination of peroxide (iodimetrically) and fat-aldehyde (Schibsted) values on fat extracted after storage at 15° C. gave values for the 'low-temperature' powders 2–4 times as high as those from the 'high-temperature' powders (Table 6). Sharp increases in peroxide values in the low- and high-temperature powders after storage at 15° C. for approximately 30–50 and 80–100 weeks respectively (Table 6) corresponded roughly with a rapid deterioration in flavour (Fig. 3). Peroxide values by the ferric

thiocyanate method were also determined at intervals during storage at room temperature. The method of Chapman & McFarlane(14) was used and proved very satisfactory, except that in order to obtain the maximum peroxide value the moisture content of all the powders had to be raised by exposure to a moist atmosphere (100% R.H.) for 48 hr. before extraction with acetone. The results are shown in Fig. 5, where the effect of raising the pre-heating temperature can readily be seen. Selecting the milk also

Table 6. *Peroxide* and fat-aldehyde values of the fat of powders stored at* 15° C. (*Cambridge samples*)

Trial no.	Nature of the dried milk	Pre-heating temp. °F.	65 weeks Tinplate cans P.V.	65 weeks Tinplate cans F.A.V.	93 weeks Tinplate cans P.V.	93 weeks Lacquered cans P.V.	104 weeks Tinplate cans P.V.	129 weeks Tinplate cans P.V.	129 weeks Lacquered cans P.V.
			Air-packed samples						
1	Ordinary, unclarified	165	14·0	30	33·0	33·4	44·0	—	99·0
2	Ordinary, unclarified	190	5·0	10	9·4	8·2	13·6	29·6	30·2
4	Ordinary, clarified	165	12·0	26	28·2	30·0	38·6	—	79·8
3	Ordinary, clarified	190	4·4	8	8·4	8·0	9·2	29·6	26·5
6	Selected, unclarified	165	8·6	18	22·0	23·4	28·7	—	67·5
7	Selected, unclarified	190	3·4	5	11·2	8·2	12·0	24·8	23·9
8	Selected, clarified	165	8·0	14	18·0	16·6	24·4	—	57·8
5	Selected, clarified	190	5·4	8	11·2	11·4	12·8	28·5	26·6
5A	Selected, clarified	190	4·2	8	—	—	12·5	22·8	—
			Gas-packed samples						
1	(3% oxygen)	165	—	—	5·0	—	3·9	6·3	—
4	(3% oxygen)	165	—	—	4·6	—	4·0	6·5	—
1	(1% oxygen)	165	—	—	2·8	—	2·5	4·0	—
4	(1% oxygen)	165	—	—	2·8	—	2·3	4·0	—

* Determined iodimetrically on fat extracted from the powders. Values recorded as m.equiv./kg. fat.

Table 7. *Effect of temperature of storage on rate of deterioration*

Method of measurement	Temperature coefficient* calculated for range	Pre-heated at the low temperature 1	4	6	8	Av.	Pre-heated at the high temperature 2	3	7	5A	Av.
Cambridge at flavour score	4·5 47–37° C.	3·0	2·8	2·6	3·3	2·9	2·2	2·3	2·3	2·3	2·3
	4·0 47–37° C.	2·5	2·4	2·3	2·7	2·5	2·0	2·1	2·1	2·2	2·1
Hannah Institute at 'off' flavour	1·0 47–37° C.	2·1	3·0	1·8	2·4	2·3	2·2	2·6	2·8	2·4	2·5
	2·0 47–37° C.	2·1	2·6	2·2	2·4	2·3	2·1	2·3	2·7	2·4	2·4
Cambridge at flavour score	4·5 37–15° C.	1·8	2·0	1·9	1·8	1·9	2·1	2·1	2·1	2·1	2·1
	4·0 37–15° C.	1·8	2·0	1·8	1·8	1·9	2·1	2·2	2·1	2·1	2·1
Hannah Institute at 'off' flavour	1·0 37–15° C.	2·2	2·3	1·8	1·7	2·0	1·9	1·8	1·8	1·8	1·8
	2·0 37–15° C.	2·1	2·2	1·8	1·8	2·0	2·0	1·9	1·8	1·9	1·9

* For the purpose of these calculations the 'temperature coefficient' is defined as the ratio of the times required for the development of a given degree of deterioration in flavour at two temperatures 10° C. apart.

resulted in lower peroxide and fat-aldehyde values, and clarification may have had a slight beneficial effect (Table 6 and Fig. 5). No appreciable chemical difference between the samples in plain and in lacquered tinplate cans could be detected after storage at 15° C., except perhaps for powder no. 7 after 93 weeks, where an appreciable difference in peroxide value (Table 6) accompanied a tasting result in which a slight but unanimous preference for the sample from the lacquered can was recorded by the six members of the panel. It

should be pointed out that after long storage at 15°C., the powders made from milk pre-heated at the high temperature showed chemical changes in the fat which in 'low-temperature' powders have usually been associated with a rather more serious deterioration in flavour (1). Part, but probably not all, of this effect may be due to a masking of incipient 'off' flavour in fat or phosphatide by the slight cooked taste imparted by the high-temperature treatment.

Relationship between stabilization and cooked flavour. It has recently been shown (10, 11, 12) that heating milk (*a*) produces sulphydryl compounds, (*b*) causes a fall in oxidation-reduction potential and (*c*) produces a boiled or cooked flavour in the milk, the two latter phenomena presumably resulting from the first. The presumptive connexion between cooked flavour and sulphydryl content on the one hand, and between

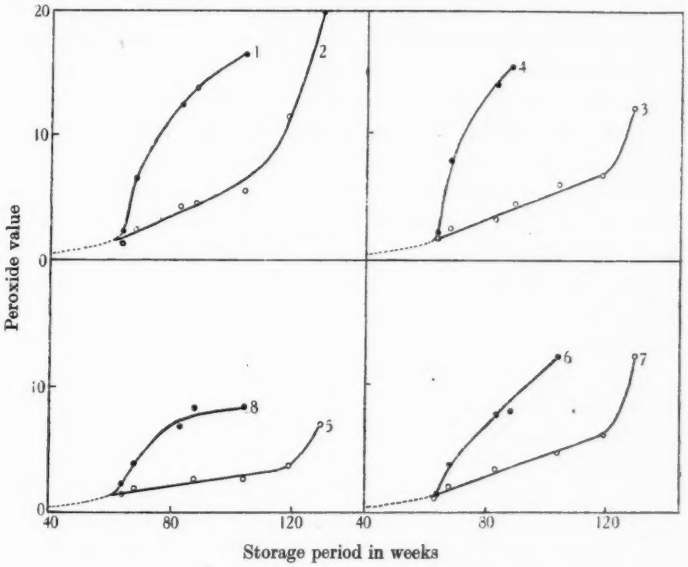

Fig. 5. Peroxide values (m.equiv./kg. powder) determined by the thiocyanate method at intervals during storage at room temperature (Hannah Institute samples)

sulphydryl content and antioxidant activity on the other, suggests that it may be impossible to stabilize milk by heating without introducing the cooked flavour. Nevertheless, the present experiments suggest that by careful control of pre-heating it will be possible to achieve a very useful measure of stabilization without too serious a production of cooked flavour.

A further point of interest in connexion with this method of stabilizing milk powder is a suggestion in some of the tasting results that the intensity of the cooked flavour in the samples from milk pre-heated at the higher temperature tends to decrease on storage, possibly as a result of gradual oxidation of the sulphide responsible. In view of the difficulty of estimating intensity of cooked (boiled) flavour over a period of time simply by tasting, without a standard for comparison, it would be desirable to check this observation by chemical means. It has, however, previously been reported that slight cooked

flavours in liquid milk, cream and butter tend to disappear on keeping, though stronger flavours are more permanent (cf. Gould & Sommer(10)).

Summary and conclusions

(1) Increasing the pre-heating temperature of the milk from 165 to 190° F. before spray-drying greatly improved the resistance of the powder to the development of tallowy 'off'-flavours on storage. From the results of accelerated tests at 47 and 37° C. a storage life (in air) of the order of 2 years could safely be predicted for the powders from milk pre-heated at the higher temperature. Confirmation has now been obtained by storage of the powders for 2½ years at 15° C. and at room temperature.

(2) Improvement in the bacteriological quality of the milk supply, and more careful control during its passage through the plant, produced some improvement in the keeping properties of powders prepared from milk pre-heated at the lower temperature, but had relatively little effect on the keeping properties of the powder when the higher pre-heating temperature was used.

(3) The shapes of the deterioration curves for the various powders showed that the effect of the higher pre-heating temperature and of improvement in the milk supply was to extend the induction period which precedes rapid deterioration. The protective effect of both factors was greater when measured at the 'slightly tallowy' or 'fairly good' stage than when comparison was made at a more advanced stage of decomposition.

(4) Removal of separator slime by use of the clarifier did not significantly increase the storage life of the powders.

(5) Pre-heating at the higher temperature did not appreciably reduce the solubility of the powder, but it produced in the milk a noticeable 'cooked' or 'boiled' milk taste. This taste was not considered objectionable, and some evidence was obtained that it tended to decrease in intensity on storage.

(6) Sulphydryl compounds were readily detectable in the dried milk pre-heated at the high, but not at the low temperature.

(7) It is suggested that the protective effect of the high pre-heating temperature is due (*a*) to production in the milk of antioxidant-active sulphydryl compounds, by the action of heat on the proteins, and possibly (*b*) to the more complete destruction of oxidizing enzymes originally present in the milk or produced therein by the growth of bacteria.

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PART II. THE EFFECT OF THE VARIOUS FACTORS UPON THE BACTERIAL (PLATE) COUNT OF THE INTERMEDIATE PRODUCTS AND OF THE FINAL POWDER

BY A. T. R. MATTICK, EDITH R. HISCOX AND E. L. CROSSLEY

INTRODUCTION

There is little literature on the effect of the factors set out on p. 123 on the qualitative and quantitative aspects of the flora of the final powder, but fairly extensive series of examinations have shown that spray-dried milk powder may contain enormous numbers of micro-organisms (1).

Milk and milk products in whatever form should not at any stage contain large numbers of micro-organisms, first, because such contamination is avoidable, and second, because the mere destruction or exclusion of pathogenic types should not preclude any endeavour to eliminate bacteria of all types during the manufacture and handling of dried milk. The results of the keeping quality tests (p. 128) show clearly that 'freshness' affects the keeping quality of dried milk powder. It is, moreover, to be remembered that for many purposes dried milk is reconstituted before use and opportunity afforded for the growth of the residual flora. Some organisms formerly regarded as harmless are now known to produce toxins and on these grounds alone it is desirable to reduce the bacterial content to a minimum at all stages. Not the least of the objects of this part of the experiment was therefore to produce a dried milk powder of the best possible bacteriological quality and, if possible, to secure data upon which standards may later be based.

Samples which were taken in the manner described (p. 120) for the estimation of keeping quality and for chemical, physical and nutritional properties, were also examined bacteriologically. This section of the general experiment was, however, concerned not merely with the numbers and perhaps the types of micro-organisms appearing in the final powder but with the raw milk supply, the milk after pre-heating, after concentrating and before spraying.

PLATE COUNTS

Samples of milk and powder were plated on yeastrel milk agar and incubated for 3 days at 37° C.

Plate counts of the raw milk were not made until the 4th phase of the experiment was reached, but the results of the methylene-blue reduction tests, which were made throughout, suggest that these counts fairly represent those of the whole of the first (control) period when ordinary milk supplies, collected once daily, were used. During the second (experimental) period the milk, which was then collected twice daily, was selected on the dairy platform by means of the 10 min. resazurin test (2). Only that which reached a standard of disk 6 or 5½ was used for drying (see Appendix). The milk that passed through the plant during this period had been cooled to about 50° F. and was of lower plate count than that used during the first period. Laboratory pasteurization of the raw milk was used as a standard for the suitability of the milk for processing and of the efficiency of pre-heating. For this purpose 10 ml. of raw milk were heated rapidly to 146° F., held at 145° F. for 30 min. and cooled.

Table 8. *Methylene-blue reduction tests and mean plate counts on yeastrel milk agar—37° C.—3 days*

Phase of experiment	Milk: Supply	Milk: Collection	Clarifier	Pre-heating temp. °F.	Raw milk: No. of samples	Raw milk: Mean reduction time hr.	Raw milk: Count per 1 ml.	Laboratory pasteurized milk (145° F. 30 min.) count per 1 ml.	Pre-heated milk count per 1 ml.	Evaporator discharge count per 1 ml.	Atomizer feed count per 1 ml.	Dried milk powder count per 1 g.
					Control period							
1, inc. 1 A	Ordinary	Once daily	Not used	165	7	1·7	—	8,474	16,827	148,380	140,380	2,877
2	,,	,,	Not used	190	6	2·25	—	5,485	133	22,866	27,173	3,726
3	,,	,,	Used	190	6	2·2	—	17,652	160	4,926	6,754	*4,117
4	,,	,,	Used	165	6	2·3	4,273,000	10,314	4,398	50,283	27,250	1,164
					Experimental period							
5, inc. 5 A	Selected	Twice daily	Used	190	10	3·55	795,000	5,320	85	3,256	2,317	562
6	,,	,,	Not used	165	6	3·7	732,300	2,245	2,027	11,569	10,668	590
7	,,	,,	Not used	190	4	4·25	343,700	8,576	165	964	409	486
8	,,	,,	Used	165	4	3·62	813,000	8,201	2,155	11,840	10,201	487

* Three faulty samples omitted (see p. 137).

Table 9. *Mean plate counts at* 37° C. *representing* 1 *ml. of milk at various stages of the process*

Phase of experiment	Pre-heating temp. °F.	From pre-heater 37° C.	From evaporator 37° C.	From atomizer feed 37° C.	Powder (immediate) 37° C.
1 (including 1 A)	165	16,800	30,000	28,000	290
2	190	130	4,600	5,400	370
3	190	160	1,000	1,300	410
4	165	4,400	10,000	5,500	20
5 (including 5 A)	190	85	600	460	56
6	165	2,000	2,300	2,100	59
7	190	165	200	80	49
8	165	2,200	2,400	2,000	49

At first only the 1/100 dilution of laboratory pasteurized milk and of milk pre-heated and concentrated in the plant was plated, but as the work progressed it was found necessary to make modifications. For the pre-heated samples the 1/10 dilution, and for the concentrated samples the 1/1000 dilution, were also included. During this series of tests the dilutions of concentrated milk were, for speed of manipulation, made by volume, and since the samples were viscous, the results were inevitably rather irregular. In a few cases, however, discrepancies between the dilutions were too great to be explained on these grounds and it was possible that they were due to the unequal distribution and the breakdown of large clumps of bacteria, but corroborative evidence was impossible to secure at the time.

A summary of the results of plate counts at 37° C. is given in Table 8, in which the geometric means of the counts of the samples taken during each phase of the experiment are given. This illustrates the reduction in plate count of the powder with the 'improvements' progressively introduced into the process of manufacture. These figures are based on the assumption, which is not strictly true, that the reconstituted milk contained 0·1 g. of powder per 1 ml.

During the third phase of the experiment three samples of powder gave unexpectedly high counts, which were found to be probably due to contamination from a blank end in the pipe leading from the atomizer feed tank to the atomizer, in which concentrated milk could lie dormant at about 100° F. These powder samples had been taken from the first quantities of powder passing through the sifter, at the beginning of the day's run, and would therefore probably contain the greater portion of any residual plant contamination. Samples of the bulk powder from these runs tested after 5 and 10 months' storage, gave no indication of unduly high plate counts. The counts of these samples were omitted in calculating the means for the freshly made powder (Table 8), but were included in the means for the plate counts after storage.

At the beginning of the control phase the cleaning and sterilizing of the plant were not really thorough, but the methods were progressively improved during this period. The calandria tubes and vapour separators were potential sources of infection and they were probably mainly responsible for the high counts in the concentrated milk samples in phases 1 and 2 of the experiment. In the second period, in which 'all-out' efforts were made to clean and sterilize the plant and only milk of a reasonably good hygienic quality was used, the danger of the existence of these foci of infection in the evaporator was greatly reduced and the plate counts of the concentrated milk were much lower.

The milk was reduced to about one-fifth of its original bulk by evaporation, and 1 g. of milk powder corresponds roughly to 10 ml. of liquid milk. In Table 9 the mean counts for concentrated milk and milk powder have been divided by 5 and 10 respectively, giving approximately the changes in the plate count of 1 ml. of liquid milk at various stages in its progress through the plant.

Very little contamination appeared to result from the passage of the milk through the evaporator once the plant had been efficiently cleaned. In addition to the reduction caused by pre-heating, there was further destruction of organisms in the drying chamber.

Influence of pre-heating temperature

From Table 9 it appears that in the production of powdered milk of low bacterial count, good plant cleaning and the use of a good milk supply were more effective than

raising the pre-heating temperature. Thus, there seems to be no essential difference between the mean plate counts of the powders in each of the four phases of the second period. The actual individual counts, however, ranged from 100 to 2360 per 1 g. of powder. The plate counts of the samples of concentrated milk taken from the evaporators and the atomizer feed tank were, however, much lower when the high pre-heating temperature was used. In actual practice, high temperature pre-heating proved of great value in reducing infection of the evaporators by thermoduric organisms derived from raw milk.

This apparent lack of discrimination between powders produced under conditions of consistently good plant management, but with the use of two distinct pre-heating temperatures, the higher of which caused a much larger destruction of organisms in the raw milk followed by smaller numbers in the concentrated milk, is due to the plating methods adopted during the experiment, which were those previously described by Crossley & Johnson (3). The plates were incubated at 37° C., but later platings of powder samples showed that incubation at 30° C. gave considerably higher counts. We found that when incubating at 37° C. a fall of temperature as little as 2° C. may result in a large increase in the plate count of milk powder, and an error in the adjustment of the incubator may have significant effects and cause large differences in results from laboratory to laboratory—a serious matter when standard methods are under consideration. The influence of slight changes of temperature is far less marked at 30° C. This matter will be discussed in a later paper.

Samples of bulk powder taken during the experiment were plated after storage at room temperature (40–70° F.) for 10–11 months using 30 and 37° C. as incubation temperatures. A fuller report of this work will also be given elsewhere. Only gas-packed samples are considered here. No doubt a number of organisms had died during this storage period, but according to Nichols (1) and Crossley & Johnson (3) the rate of mortality was less for gas-packed than for air-packed samples. The means of the counts are given in Table 10, in which these figures are compared with those obtained at the time of the experiment. One tin from each batch of powder was used since serial platings of a number of tins of selected batches had shown that although, as was to be expected from the method of packing, slight but significant differences might be found in the counts of samples from individual tins in each batch, in general the differences were not large enough to obscure differences between the batches. The counts for the plates incubated at 37° C. were of the same order as those recorded for the fresh powder at the time of the experiment, but the counts at 30° C. were much larger. The counts for samples A and B, powder from the same plant made during the following spring and plated 17 days after manufacture, show that the differences between counts at the two incubation temperatures existed in the freshly made powder and were not the result of storage. This was also true of samples of powder from an entirely different plant tested within 4 days of manufacture.

With the larger counts obtained by incubation at 30° C. a greater differentiation between the various phases of the experiment became apparent, and the effect of the various factors may be more clearly assessed. Although the figures for the plate counts at 37° C. suggest that good plant cleaning and sterilization, and a good milk supply are of greater importance than the pre-heating temperature in the production of powder of low bacterial count, the figures for plate counts at 30° C. showed that pre-heating at

Table 10. *Mean plate counts on yeastrel milk agar*

After storage for 10–11 months

Table 10. *Mean plate counts on yeastrel milk agar*

Phase of experiment	Milk supply	Collection	Clarifier	Pre-heating temp. °F.	No. of samples	At time of experiment: Milk pasteurized in lab. inc. 3 days at 37°C. per 1 ml.	At time of experiment: Milk after pre-heating in plant inc. 3 days at 37°C. per 1 ml.	Dried milk powder inc. 3 days at 37°C. per 1 g.	After storage for 10–11 months in gas-packed tins at temp. of 40–70° F. Dried milk powder: Inc. 5 days at 37°C. per 1 g. powder	After storage for 10–11 months in gas-packed tins at temp. of 40–70° F. Dried milk powder: Inc. 5 days at 30°C. per 1. g. powder
1, inc. 1A	Ordinary	Once daily	Not used	165	7	8,500	16,800	2,900	9,000	175,000
2	,,	,,	Not used	190	6	5,500	130	3,700	1,600	9,700
3	,,	,,	Used	190	6	17,600	160	4,100	1,000	13,600
4	,,	,,	Used	165	6	10,300	4,400	1,200	1,700	296,000
1A	,,	,,	Not used	165	2	2,300	22,400	2,800	5,700	63,300
5, inc. 5A	Selected	Twice daily	Used	190	10	5,300	85	560	830 (860)*	6,000 (3,900)*
6	,,	,,	Not used	165	6	2,200	2,000	590	1,200	92,000
7	,,	,,	Not used	190	4	8,600	160	490	420	2,900
8	,,	,,	Used	165	4	8,200	2,100	490	1,500	46,800
5A	,,	,,	Used	190	4	3,800	55	290	490	2,800
			Freshly made powder (1943)						17 days after manufacture: Inc. 37°C.	17 days after manufacture: Inc. 30°C.
A	Ordinary	Once daily	Not used	165	1	—	—	—	1,300	102,000
B	,,	,,	Not used	190	1	—	—	—	1,400	15,700

* The figures in brackets exclude high figures obtained on the first day of the change-over of milk supply, when many minor 'accidents' occurred.

Table 11. *Number of samples in which coliform organisms were found*

Phase of experiment	Milk supply	Collection	Clarifier	Pre-heating temp. °F.	Raw milk: +0·01 ml.	Raw milk: +0·001 ml.	Raw milk: +0·0001 ml.	Laboratory-pasteurized milk +10 ml.	Pre-heated milk +10 ml.	Evaporator discharge: +1 ml.	Evaporator discharge: +0·1 ml.	Evaporator discharge: +0·01 ml.	Atomizer feed: +1 ml.	Atomizer feed: +0·1 ml.	Atomizer feed: +0·01 ml.	Powder: +1 g.	Powder: +2 g.
1 (inc. 1A)	Ordinary	Once daily	Not used	165	—	3	4	—	—	2	2	—	1	3	2	1	—
2	,,	,,	Not used	190	—	2	4	—	—	—	1	1	2	1	1	1	—
3	,,	,,	Used	190	—	—	6	1*	—	—	—	—	2	1	—	1	—
4	,,	,,	Used	165	—	1	5	—	—	—	—	—	2	—	—	2	—
5	Selected	Twice daily	Used	190	1	4	3	—	—	1	—	—	—	—	—	—	1
6	,,	,,	Not used	165	—	4	2	—	3*	—	—	—	—	—	—	—	—
7	,,	,,	Not used	190	—	2	1	—	—	—	—	—	—	1	—	—	—
8	,,	,,	Used	165	1	2	1	—	—	—	—	—	—	—	—	—	—

* Heat-resistant strains.

190° F. produced a powder of much lower count than pre-heating at 165° F. even during the second or experimental period when the plant was at its maximum cleanliness and a selected milk supply was used. During the fourth phase of the first (control) period, the plate counts at 30° C. were higher than had been expected, since the cleaning of the plant had then reached its best. The high bacterial content was probably due to under-heating of the milk, as is suggested by the phosphatase tests (Table 13) for the pre-heated milk during that period. The temperature of the pre-heater was not automatically controlled, and notes made during the runs at this period of the experiment show that the operator on duty during the morning (morning powders gave the highest counts) of that period was not meticulously careful of detail and allowed undue fluctuations in the temperature of the pre-heater. The organisms that were most sensitive to these fluctuations of temperature were, presumably, those that did not grow well at 37° C., and it was therefore only the plate counts at 30° C. that showed the connexion between careless plant control and the final bacteriological quality of the powder. In view of the probable effect of bacterial enzymes or products of growth on keeping quality, it seems desirable to plate at 30° C. as well as at 37° C.

Influence of milk supply

When the second period of the experiment was reached and selected milk supplies of considerably lower plate count were used, there was an immediate decrease in the total count (at 30 and 37° C.) of the powder. The mean counts as shown in Tables 8 and 10 did not, however, show any marked decrease in the mean numbers of thermoduric organisms surviving laboratory pasteurization, nor suggest that this milk was more suitable for processing. It should be said that no attempt could be made to improve conditions of production at the farms owing to an outbreak of foot and mouth disease in the area. Examination of the individual counts, however, revealed that whereas in the first period 48% of the samples showed counts of not more than 10,000 and 17·5% counts of not more than 3000 thermoduric organisms per 1 ml., the corresponding figures for the selected milk supplies were 71 and 54% respectively. The high mean counts of groups 5, 7 and 8 were due to the presence of three samples with high thermoduric counts. Thus, though no doubt the satisfactory cleanliness of the plant was of value in the production of powder with a low plate count during the second period, the use on most occasions of a milk supply of better quality was in fact an important contributory factor.

Use of clarifier

There is no good evidence in the counts for the individual samples, or in the means in Tables 8 and 10 that the use of a clarifier significantly reduced the number of organisms surviving laboratory pasteurization or pre-heating in the plant, or exerted any constant influence on the plate count of the powder. The chief function of the clarifier is probably the removal of slime and foreign material which might act as catalysts for chemical reactions during storage.

COLIFORM ORGANISMS

These organisms, which are not truly heat resistant and therefore useful as indicators of contamination, were detected by inoculation of measured quantities of the sample into McConkey broth and incubating at 37° C. for 2 days. For the laboratory-pasteurized milk, and in the second period of the experiment for the pre-heated milk, 10 ml. quan-

tities were used, 1 ml. for the concentrated milk and two quantities of 10 ml. (1 g. powder) each of the reconstituted dried milk powder (Table 11). Presumptive positives were confirmed or disproved by plating and isolation of cultures. Coliform organisms in significant numbers were present in all samples of raw milk with one possible exception in which they were absent from 1/100 dilution (the lowest tested). During the control period when the ordinary milk supply, collected once daily, was used, they were found in 1/10,000 dilution (the highest dilution tested) in twenty-one samples and in 1/1000 dilution in four samples. During the experimental period, when selected milk supplies, collected twice daily, were used, there was some improvement though even during this period in only one sample were they not found in 1/100 dilution, and in only four not beyond the 1/100 dilution. In seven of the twenty-four samples they were found in 1/10,000 dilution (the highest dilution tested). It may therefore be concluded that although the selected supplies were better than the unselected, they were in fact not very good.

Coliform organisms were readily destroyed by laboratory pasteurization and by the pre-heating process. Strains resisting this treatment were found on four occasions only, once in 10 ml. samples of ordinary milk pasteurized in the laboratory at 145° F. for 30 min., and three times in 10 ml. samples of selected milk pre-heated at 165° F. 'False' positives were found twice in the laboratory pasteurized and once in the pre-heated milk, but the causal organisms were not identified.

During the first phase of the control period coliform organisms were found in almost all samples taken at the outlet from the evaporator and from the atomizer feed pipe, no doubt as a result of residual contamination in this part of the plant. On the 5th day the evaporator was given a special cleaning and afterwards kept in good order. Of the forty samples subsequently taken from the evaporator outlet only one showed the presence of coliform organisms in 1 ml.

Coliform organisms were found in 1 g. quantities of powder (10 ml. of reconstituted milk) five times during the control period. Three of these powders were made from milk pre-heated at 165° F. and, since it is known that the temperature fluctuated slightly, the high phosphatase contents of the powders suggest that the presence of the coliform organisms was due to the inclusion of slightly underheated milk. The other two samples, produced during the early phases of the experiment, were from milk pre-heated at 190° F. The infection here was probably due to extraneous contamination such as might result from the distribution of residues of loose powder over the sifter at the beginning of the day's run (p. 143). Both were morning samples and, moreover, showed unduly heavy contamination with moulds. During the experimental period coliform organisms were found only once, in 2 g. of the powder from the final run.

THERMOPHILIC ORGANISMS

These were estimated by plating on yeastrel milk agar and incubating at 63° C. for 24 hr. Few thermophilic organisms were found in the raw milk and few were found in the finished powder after the first few days of the experiment, i.e. when the methods of plant cleaning had been improved and there was less danger of organisms accumulating in the evaporator where temperature conditions were favourable for the growth of thermophiles. The counts, which were always less than 100 per 1 ml. (frequently no colonies were found on 1/10 plate), were too indefinite to warrant any attempt at correlation with the various factors introduced during the experiment (Table 12).

Table 12. *Incidence of thermophilic organisms, moulds and spore-forming anaerobes*

Phase of experiment	Milk supply	Collection	Clarifier	Pre-heating temp. °F.	No. of samples tested	Thermophilic organisms		Moulds		Anaerobes	
						<100 per 1 ml.	>100 per 1 ml.	No. of positive samples	Counts of positives per 1 g.	No. of positives	Type
1	Ordinary	Once daily	–	165	7	6	1	2	220 200	4	*Cl. butyricum* (3) *Cl. putrificum* (1)
2	,,	,,	–	190	6	3	3	3	90 40 10	0	—
3	,,	,,	+	190	6	6	0	2	570 20	0	—
4	,,	,,	+	165	6	6	0	1	20	1	*Cl. butyricum*
5	Selected	Twice daily	+	190	10	6*	0	1	10	0	—
6	,,	,,	–	165	8	6	0	1	10	0	—
7	,,	,,	–	190	6	4	0	0	—	0	—
8	,,	,,	+	165	—	Not tested		0	—	1	*Cl. welchii*

* Only six samples tested.

YEASTS AND MOULDS

These were estimated by plating on whey agar (*p*H 3·5) and incubating at 22° C. for 4 days. Only the powder was tested. No yeasts were found, but moulds were present in appreciable numbers in five samples taken during the first three phases of the experiment. Judging by the generally low heat resistance of mould spores it is unlikely that they had survived the drying process. They probably resulted from after-contamination. As was mentioned in the section on the sampling of the powder, the sifter was exposed to air contamination, and early in the experiment no real effort was made to avoid draughts and floating dust particles. Since these samples were taken directly from the sifter outlet, air contamination should not have been heavy enough to account for such numbers as 570 or 220 per 1 g. of powder found in two cases. The more probable source of the contamination was the residues of loose powder which it had been customary to pour on the sifter for distribution with the fresh powder at the beginning of the day's run. On some occasions this was noticed and the time of sampling was postponed in the hope that this extraneous powder would be eliminated before the sample was taken. The mould-infected samples were all morning samples taken near the beginning of the day's run. The danger of this source of contamination had been removed by the time the experimental period was reached. Weather conditions may also have been a factor in the distribution of mould spores.

ANAEROBIC SPORE-BEARING BACTERIA

These tests were confined to the milk powder. Five ml. of reconstituted milk were inoculated into milk peptone meat medium (4), heated at 80° C. for 15 min. and incubated at 37° C. for 3 days. Only spores would be detected by this method.

During the control period, when the ordinary milk supplies were used, *Cl. butyricum* was found in four and *Cl. putrificum* in one sample. During the experimental period, when selected supplies were used, *Cl. welchii* was found in one sample. The infrequent occurrence of these organisms suggests that plant contamination was not their source; they were probably derived from the milk supply and, being in the highly resistant spore form, were unaffected by the process.

GENERAL OBSERVATIONS ON FLORA

During the experiment, time could not be found for detailed identification of the general flora of the milk powder. Some of the less numerous types were identified and assessed by the special tests just described, and a rough estimate of the major flora was made by inspection of colony forms on the plates. These indicated that the flora consisted mainly of streptococci, micrococci and spore-bearing rods. As the cleanliness of the plant improved, the incidence of streptococci declined and in some cases micrococci constituted almost the entire flora. The pre-heating temperature also influenced the types as well as the numbers of organisms. With a pre-heating temperature of 165° F. and a clean plant, certain strains of micrococci were dominant, but with 190° F. strains of streptococci tended to predominate over the micrococci. Spore-bearing rods also constituted a significant part of the flora.

The flora is being studied more fully in samples of the stored powder and in the main the findings support the preliminary survey based on the inspection of plates. There

was one group of bacteria which was, however, overlooked until plates incubated at 30° C. were examined. These were small rods, usually weak acid-producers in milk and growing feebly if at all at temperatures above 35° C.—probably the microbacteria of Orla-Jensen (5) (corynebacteria of Gibson (6)). In the 'low-temperature' powders they constituted so high a percentage of the flora that they far outnumbered all other types. In the 'high-temperature' powders they were less numerous, and the streptococci, micrococci and sporing rods became therefore of greater relative significance.

Summary

During the control period, when cleaning and plant management were being improved, the plate counts, at 37° C., per 1 g. of powder were significantly higher than those recorded during the experimental period when control and cleaning were good and the milk supply was improved. The plate counts finally recorded (and since sustained with this plant) amounted to only a few hundreds per 1 g. of powder.

Examinations of samples of some powders after several months' storage showed that plate counts made after incubation at 30° C. were higher than those at 37° C. The influence of pre-heating at 190 instead of 165° F., accompanied by improvements in plant cleaning and sterilization and in the quality of the milk supply, which was doubtfully reflected, if at all, by plate counts at 37° C., was clearly evident in the plate counts made at 30° C. It is therefore recommended that in any method of standardization and for purposes of plant control, the inclusion of plate counts at 30° C. should be considered.

The importance of maintaining accurate incubation temperatures in making plate counts of dried milk at 37° C. has been emphasized.

The factors which determined the plate count of dried milk appeared to be, in the order of their importance: (1) plant cleanliness and sterility, (2) pre-heating temperature, (3) bacteriological quality of the raw milk supply, which in its turn affected the hygienic condition of the plant.

A preliminary examination of the flora surviving in the dried milk powder showed it to comprise streptococci, micrococci and spore-bearing organisms. With improvements in plant cleaning the relative numbers of streptococci declined. The temperature of pre-heating influenced type as well as numbers of organisms. With a pre-heating temperature of 165° F. and a clean plant, micrococci predominated over the streptococci, whereas when the pre-heating temperature was 190° F. streptococci predominated and spore bearers were relatively numerous.

When plates were incubated at 30° C. it was observed that microbacteria, hitherto apparently not reported in dried milk powder, were found to predominate over all other types of organisms.

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PART III. CHEMICAL, ENZYME AND VITAMIN DETERMINATIONS

A. ACIDITY, AMYLASE, PHOSPHATASE AND LIPASE

BY E. L. CROSSLEY

B. VITAMIN STABILITY: WITH OBSERVATIONS ON FLAVOUR DEVELOPMENT IN THE RECONSTITUTED MILKS

BY S. Y. THOMPSON AND S. K. KON

The native enzymes of milk, with those of the bacterial population, may by their action initiate or promote chemical changes which may in their turn affect the keeping or other properties of the dried milk. The acidity, here calculated as lactic acid, may also have its effect.

The question of the stability of the vitamins A, B and C, which are subject to oxidation and of riboflavin is also obviously important.

The opportunity was therefore taken of exploring, as far as possible, the facts relating to some of the enzymes, the vitamins and the acidity as they were affected by the variations in the factory procedures.

A. ENZYME DETERMINATIONS

Enzyme determinations were made on pre-heated milk from the plant and on powders reconstituted by dissolving 10 g. powder in 90 ml. distilled water at 40° C., with thorough shaking. Samples from thirty-three runs were tested.

For the phosphatase tests the standard Kay and Graham technique, which appeared to suit the purpose quite satisfactorily, was used.

The presence of amylase in milk powder seems unlikely to influence its keeping quality, but it was considered that the information might yield useful indications of the effects of heating.

A mixture of 10 ml. milk and 0·5 ml. sodium chloride-starch solution (1 g. soluble starch, 50 g. sodium chloride dissolved in 250 ml. water) was incubated for 4 hr. at 30° C., followed by addition of 2 ml. of 50:50 chloroform-glacial acetic acid mixture. The solution was centrifuged at high speed for 15 min., the clear fluid poured off, and one drop of iodine solution (0·6346 g. iodine, 30 g. potassium iodide, 100 ml. water) added to it. A blue colour indicated destruction of amylase by heat, whilst a yellow colour indicated strong enzyme action. Reconstituted powders yielded colour shades somewhat different from those of control samples of raw milk and raw milk heated to various temperatures, but interpretation of the results presented no difficulty.

Attempts were made to estimate the lipase content of powders by a modification of the method described by Mattick & Kay(1) for the determination of tributyrinase in raw milk. The method consists essentially of incubation at 37° C. for 5 hr. of a definite volume of reconstituted milk with a known quantity of tributyrin in a buffer solution of sodium diethyl barbiturate at *p*H 8·5. The butyric acid formed is steam distilled from the acidified reaction mixture and titrated with 0·01 *N* NaOH. A control value is obtained by steam distillation of a portion of the acidified mixture before incubation; the titration figure thus obtained is deducted from the final figure after incubation.

The technique employed was very similar to that described by Mattick and Kay, except that rather larger reaction mixtures were used. Phosphoric acid was used for

acidification of reaction mixtures to *p*H 2·0 before steam distillation; some experiments with sulphuric acid yielded very high values. A standard distillation apparatus, fitted with ground glass joints was employed. Great care was taken to ensure sterility of all reaction flasks, pipettes, etc. in order to avoid any danger of bacterial multiplication during the incubation period, although this danger was slight in view of the short incubation time.

Nevertheless, reproducibility of results was not good for powder samples, and no great accuracy is attributed to the figures obtained. Control samples of raw milk yielded quite satisfactory results; the values obtained agreed closely with figures given by Mattick and Kay. It was evident that some unknown factor influenced the results, and the method was not strictly applicable to milk powder.

Indeed, it became obvious during this work that further investigation of the general enzyme content of milk powder should be undertaken, bearing in mind that methods of determination employed for raw milk may not be suited to milk powder.

Phosphatase

The milk was pre-heated in the plant at either 165 or 190° F. for about 20 sec. It was caught in a small balance tank before passing to the evaporator and thus had an additional holding period of about 3 min. at about 163 or 188° F. respectively.

Even the less severe treatment was equivalent to rather more drastic heating than holder pasteurization at 145° F. for 30 min. For this reason the Kay & Graham phosphatase standard of 2·3 blue units was regarded as the maximum value which should be accepted. During one 'low-temperature' period (trial 4), however, all the milk samples examined showed evidence of inadequate heat treatment and one sample gave a reading of 8·0 blue units (Table 13). The cause was traced to variations in the temperature of the milk flowing through the tubular heater. Unsatisfactory tests were not observed in the other low-temperature trials, but this experience suggests that the margin of safety in this temperature region is inadequate unless automatic controls are used.

Under the operating conditions studied it would have been impossible to collect powder samples corresponding exactly to given samples of milk, although sampling was timed to achieve this as nearly as possible. Nevertheless, it may be useful to record that powder samples usually yielded rather lower phosphatase readings than did the samples of pre-heated milk (Table 13). In spite of this, the four samples of pre-heated milk in trial 4 which showed high phosphatase readings yielded three powders wherein the phosphatase test still exceeded 2·3 blue units. It may therefore be assumed that, when low temperature pre-heating is employed, inadequate heat treatment might be detected by subsequent phosphatase tests on the powder. This matter is of importance since previous work of Crossley & Johnson(2) has shown that bacteria which survive pre-heating may also survive spray drying, so that the pre-heating process itself must be sufficient to destroy pathogenic organisms.

Pre-heating at 190° F. is obviously outside the useful range of the phosphatase test, and in any case there is little likelihood of survival of the usual pathogenic organisms. The results recorded in Table 13 indicate that lower phosphatase values were obtained from 190 than from 165° F. samples, as would be expected.

Table 13. *Chemical data*

Trial	Run	Milk collection	Pre-heating temp. °F.	Clarifier	Acidity % lactic acid	Phosphatase blue units		Amylase		Sulphydryls	
						Milk	Powder	Milk	Powder	Milk	Powder
1	27–1	Once daily	165	Not used	1·13	.	2·3	.	–	.	–
	28–1				1·13	.	1·6	.	–	.	–
	28–2				1·13	.	2·3	.	Trace	.	–
	29–1				1·04	.	2·4	.	–	.	Trace
	29–2				1·13	.	2·2	.	+	.	Trace
1A	9–1				1·08	.	2·2	.	–	–	–
	9–2				1·04	.	.	.	.	–	–
				Mean	1·12						
2	30–1	Once daily	190	Not used	1·13	.	1·8	.	–	.	+ +
	30–2				1·22	.	.	.	.	.	+ +
	31–1				1·08	.	2·1	.	–	.	+
	31–2				1·13	.	.	.	.	.	+
	1–1				1·22	.	1·8	–	–	.	+
	1–2				1·22	.	.	.	.	.	.
				Mean	1·17						
3	3–1	Once daily	190	Used	1·13	.	2·2	.	.	+	+
	3–2				1·08	.	.	.	.	+ +	+ +
	4–1				1·08	2·2	1·9	–	–	+ +	+ +
	4–2				1·13	.	2·0	–	–	.	+ +
	5–1				1·08	.	.	–	–	.	+ +
	5–2				1·17	2·1	1·8	–	–	+ +	+ +
				Mean	1·11						
4	6–1	Once daily	165	Used	1·22	8·0	2·9	+	Trace	–	–
	6–2				1·17	2·6	2·2	+	Trace	–	–
	7–1				1·13	2·9	2·8	+	Trace	Trace?	–
	7–2				1·17	.	.	+	.	–	–
	8–1				1·22	3·1	2·6	+	–	–	–
	8–2				1·13	.	.	+	Trace	Trace	Trace
				Mean	1·17						
5	10–1	Twice daily	190	Used	0·99	1·9	1·4	.	.	.	+
	10–2				0·99	.	.	.	.	.	.
	11–1				0·95	1·9	1·5	–	–	+ +	+ +
	11–2				0·95	1·9	.	.	.	.	.
	12–1				0·99	1·9	1·6	–	–	+	+ +
	12–2				0·95	1·8	.	–	–	+	+
5A	21–1				1·03	1·4	1·8	–	–	+ +	+ +
	21–2				1·08	.	.	.	.	.	+ +
	22–1				1·17	.	1·8	.	.	.	+ +
	22–2				0·99	.	.	.	.	.	.
				Mean	1·01						
6	13–1	Twice daily	165	Not used	0·95	.	1·5	.	Trace	.	Trace
	13–2				0·90	2·1	2·1	Trace	Trace	–	–
	14–1				0·87	2·1	1·7	+	Trace	Trace	–
	14–2				0·80	.	.	.	.	–	.
	15–1				0·85	1·8	.	+	.	–	.
	15–2				0·90	.	.	.	.	.	.
				Mean	0·88						
7	17–1	Twice daily	190	Not used	0·94	1·5	1·2	.	.	.	+ +
	17–2				0·90	.	2·1	.	.	+ +	+ +
	18–1				0·90	.	0·9	–	–	.	+ + +
	18–2				0·99	.	.	.	.	+ +	+ +
				Mean	0·93						
8	19–1	Twice daily	165	Used	1·08	1·5	1·4	+	Trace	+	–
	19–2				1·08	2·2	.	+	.	+	–
	20–1				1·03	1·7	1·4	+	Trace	+	–
	20–2				1·08	.	1·2	+	.	Trace	–
				Mean	1·07						

N.B. In the coding of samples '1' denotes a.m., '2' denotes p.m. samples.

Amylase

Amylase was always present in milks pre-heated at 165° F. and was usually just detectable in the corresponding powders. Milks pre-heated at 190° F., and the corresponding powders, yielded uniformly negative results (Table 13).

Since this work has shown that high temperature pre-heating is advantageous for a variety of reasons, the amylase test may be of some value as a control method. Whilst further investigations of intermediate temperature ranges would be desirable, it is clear that a satisfactory heat treatment should destroy amylase.

Lipase

Owing to the difficulties encountered in the determination of lipase in milk powder, no reliable data are as yet available and the results of work now in progress will, it is hoped, be reported later. It may, however, be stated with some certainty that at least traces of lipase were present in all the milk powders, and the available evidence seems to show that spray drying in itself does not completely destroy lipolytic enzymes. Considerable complication arises from the fact that in addition to lipase native to the milk supply, lipolytic bacteria may be present at all stages of the manufacturing process.

Acidity

Powder acidity was determined by titration of the reconstituted milk with 0·1 *N* NaOH, using 0·5% phenolphthalein as indicator. Results were expressed as per cent lactic acid.

Milk collected twice daily yielded powders of significantly lower acidity than normal milk collected once daily (Table 13). The normal milk gave quite satisfactory results, but very low values were obtained by collecting milk twice daily. It should be added that acidity values considerably greater than those obtained during the 'once daily' period are quite common in ordinary factory practice.

Very consistent results were observed throughout the 'once daily' collection period. During the 'twice daily' period, acidities appeared to be lowest when the clarifier was not used.

Since these milks were dried within a few hours of milking, and the worst supplies were eliminated, the possibility of any considerable developed acidity is slight. Clarification might, however, favour acid production by breaking up bacterial clumps.

Sulphydryl determinations

Tests for the presence of sulphydryls (see Part I, p. 124) were carried out on both the pre-heated milk and the fresh powder. The results are given in Table 13 and accord with those given on pp. 124–5.

SUMMARY

Phosphatase determinations on milk pre-heated at 165° F. show quite clearly that, under the conditions of the experiment, this temperature, with its holding time of approximately 20 sec., was insufficient to guarantee a maximum of 2·3 L.B.U., which was adopted as the standard. Several of the readings recorded significantly exceeded this figure. With a pre-heating temperature of 190° F. no reading exceeded 2·0 and the majority were substantially less. The higher figures recorded for the milk pre-heated at 165° F. were reflected in the values for phosphatase (which in several instances exceeded

2·6 L.B.U.) for the dried milk powder. With powders prepared after heating at 190° F. all phosphatase values were less than 2·3 L.B.U. It appears from this that it is necessary to control accurately the temperature of pre-heating and that temperatures in the region of 165° F. for approximately 20 sec. in the type of heater used in this plant are not high enough to guarantee freedom from pathogenic organisms. In the absence of automatic control, moreover, it seems that unless phosphatase is destroyed in the pre-heating period it is likely to survive the relatively high temperatures of the air in the drier (about 170° C.).

Further evidence of the survival of enzymes is provided by the observations recorded for amylase. This was consistently found to be present in milk pre-heated at 165° F. and traces remained in the dried milk powder. When the milk was pre-heated at 190° F. amylase could not be demonstrated.

The presence of sulphydryls in stored powder samples has been referred to in Part I, but it may be mentioned here that with a pre-heating temperature of 165° F. only traces were detected in the milk and in the corresponding freshly made powders, whereas at 190° F. both milk and powder gave consistently positive results. The results obtained at the time of manufacture therefore agree with those observed for stored samples.

REFERENCES

(1) MATTICK, E. C. V. & KAY, H. D. (1938). *J. Dairy Res.* **9**, 58.
(2) CROSSLEY, E. L. & JOHNSON, W. A. (1942). *J. Dairy Res.* **13**, 5.

B. VITAMIN STABILITY AND FLAVOUR TESTS

Observations on milk, spray dried in the plant used in the present study, have already been made by Henry, Houston, Kon & Osborne (1) and Houston, Kon & Lea (2). They found in drying no loss of vitamin A, carotene and riboflavin, a 10% loss of vitamin B_1 and a 20% loss of ascorbic acid. Storage for 12 months in gas pack at ordinary temperature was without effect on the carotene, vitamin A and riboflavin, but vitamin C decreased by 20%. The effect on vitamin B_1 was not measured in that experiment (1). In unpublished work Houston *et al.* (2) showed that ascorbic acid, carotene and vitamin A decreased in those samples of spray-dried milk which were stored with access to air, whereas an inert-gas pack prevented loss of these factors. Again riboflavin proved quite stable in air- or gas-packed powders, but vitamin B_1 showed a decrease in all samples.

In the present investigation it was not possible to measure the vitamin content of the original milks, and the two heat treatments could not be run simultaneously from the same bulk of milk. The natural variation in the vitamin content of raw milk (Kon (3)), must be borne in mind when making comparisons of the values obtained at the initial sampling of the dried powders, although the size of the bulk from which the powders were made and the short time intervals between the samplings would considerably reduce this variation.

The tests on vitamins were planned to study the effect of the two pre-heating temperatures, of gas versus air packing, and of ordinary versus selected supplies of milk. Table 13 gives details of the samples of dried milk powder examined. To make the task manageable the numbers had to be limited, and the most widely divergent trials were chosen for comparison.

General

The gas-packed samples were prepared under ordinary commercial conditions. The seams of the tins were lacquered. On arrival at Shinfield the samples were stored in an unheated room probably simulating closely warehouse conditions. It should be noted that the powders stored at Shinfield were left in the 1 lb. tins in which they were packed at the factory, whereas those dealt with at the Hannah Institute and at Cambridge were repacked after an interval. It was subsequently found by Dr C. H. Lea that many of the gas-packed 1 lb. tins were leaky. The inclusion of such leaky tins in comparisons between gas and air pack would bias the results against the gas pack. We are unable to gauge the extent of this effect, but it could only strengthen the view expressed later in the paper regarding the superiority under certain conditions of gas pack over air pack. For each assay one tin from each run in any one trial was taken for bulking and a sample was obtained after thorough mixing. Measurements of vitamins and the assessment of flavour scores were carried out on milks reconstituted by pouring 62·5 g. of powder into 452·5 g. of distilled water at 40° C. and whisking vigorously for a few seconds with a fork. The reconstituted milks were immediately placed in the dark and sampled as soon as possible. As a check on the accuracy of reconstitution, determinations of fat and total solids were carried out on each reconstituted sample.

Ascorbic acid

The reduced form was estimated in milk serum obtained by precipitating the proteins by the method of Knight, Dutcher & Guerrant (4) modified, to ensure better precipitation of the proteins, by using trichloroacetic acid in the place of concentrated hydrochloric acid. To 20 ml. of a mixture of equal parts of 10% sodium metaphosphate and 8% trichloroacetic acid 20 ml. of milk were added and an aliquot of the filtered serum was titrated with the indophenol reagent. The total ascorbic acid was estimated after H_2S treatment of the milk serum as described by Kon & Watson (5). It must be borne in mind that sulphydryl and other reducing substances formed at the higher temperature could affect the indophenol titration, but according to Doan & Josephson (6) most of these reducing substances are removed on precipitating the protein.

Vitamin B_1 and riboflavin

These were measured fluorimetrically as described by Houston, Kon & Thompson (7) and by Henry, Houston, Kon & Thompson (8).

Vitamin A and carotene

The fat from 50 ml. of milk was extracted by the method of Olson, Hegsted & Peterson (9). The fat extracts were, however, washed twice with water and dried over anhydrous sodium sulphate before evaporation of the solvent. The fat obtained was weighed, saponified and extracted as described by Gillam, Henry & Kon (10). Readings were made using a photoelectric spectrophotometer (Thompson (11)) calibrated against pure β-carotene and a vitamin A concentrate of known potency. Vitamin A was measured by the $SbCl_3$ reaction.

Taste tests

These were carried out by one of us on the same samples of reconstituted milk as were used for the vitamin tests.

Results

The main object of our work was to study (*a*) the direct effects of different pre-heating temperatures on the vitamin content of milk, (*b*) the effects of these temperatures on the stability of these vitamins during storage, particularly in view of the possible formation of sulphydryl and other reducing substances at the higher pre-heating temperatures.

(*a*) *Direct effect.* Table 14 shows the results obtained in all the tests. As previously stated (p. 149), it is not easy to assess to what extent any differences observed at the first test were due to variations in the original raw milk, and to what extent they had been caused by treatment and unavoidable storage. However, the air- and gas-packed samples are strictly comparable. In some of the trials the collection of powders extended over periods of up to 13 days. These powders were all deemed to have been prepared on 9 August, the middle of the drying period, and at the first test on 20 and 23 November 1942 the milks were 3½ months old. Though strict comparison is not possible between samples pre-heated to different temperatures, the initial contents of vitamins A, B_1 and C and of carotenoids and riboflavin in the samples pre-heated to 190° F. are so similar to those of samples heated to 165° F. that it appears very likely that the higher temperature had no deleterious effects.

At the initial test it was found that the reduced ascorbic acid was 27% higher in the selected supply samples than in the ordinary supply samples. A possible explanation may be the short time interval between production and drying of this type of milk. Total ascorbic acid was not measured on this occasion in the selected supply samples. Carotene and vitamin A were both higher in the gas-packed samples than in corresponding air-packed samples. Aneurin and riboflavin were the same in all samples.

Taste tests carried out at this time, 6 hr. after reconstitution (Table 15), showed that the heat treatment was the main factor in determining flavour, as both air- and gas-packed samples pre-heated to 165° F. were slightly tallowy, whereas the air- and gas-packed samples pre-heated to 190° F. had an agreeable slightly cooked flavour. Though flavour is largely a matter of personal preference, our observations agree with those of the drying-plant staff who indicated a majority preference for the powders prepared from milk pre-heated to 190° F., which were considered to possess a rather pleasing characteristic flavour.

(*b*) *Effect of storage.* Each type of milk was assayed three times. The initial test was on 20 and 23 November 1942, the second test on 18 and 19 February and the final test on 23 and 25 August 1943. The results are given in Table 14 and also in Figs. 6–8.

Ascorbic acid decreased to some extent during storage in all samples (Fig. 6). After 1 year's storage the greatest loss found was 50% of the total ascorbic acid content of the ordinary supply, air-packed, low-temperature sample. The reduced ascorbic acid showed more variations than the total. The loss was greatest in the air-packed low-temperature samples and least in the gas-packed high-temperature samples. All gas-packed samples contained more ascorbic acid than air-packed, regardless of heat treatment.

Vitamin B_1 and *riboflavin* showed no change after 12 months' storage.

Vitamin A and carotenoids. Figs. 7 and 8 and Table 14 show clearly that gas-packed samples lost less vitamin A and carotenoids, regardless of heat treatment, than air-packed samples and that the high-temperature process gave better protection to these factors than the normal-temperature process. The best protection was given by a com-

Table 14. *Vitamin tests on spray-dried milk powder*

Trial	Milk supply	Collection	Pre-heating temp. °F.	Clarifier	Date of testing	Packing	Ascorbic acid μg./g. powder		Riboflavin μg./g. powder	Aneurin μg./g. powder	Carotenoids μg./g. powder	Vitamin A i.u./g. powder
							Reduced	Total				
1 and 1A	Ordinary	Once daily	165	Not used	Nov. 1942	Air	76	110	14·1	2·51	1·60	6·40
					Feb. 1943		65	102	12·7	2·97	1·49	6·20
					Aug. 1943		51	57	12·8	2·54	0·92	4·64
					Nov. 1942	Gas	77	120	13·2	2·65	1·68	7·30
					Feb. 1943		94	110	14·3	2·77	1·63	6·70
					Aug. 1943		89	97	13·7	2·65	1·55	5·76
2	Ordinary	Once daily	190	Not used	Nov. 1942	Air	73	100	12·5	2·46	1·56	6·50
					Feb. 1943		81	109	13·8	2·93	1·59	6·80
					Aug. 1943		79	82	13·2	2·68	1·40	5·60
					Nov. 1942	Gas	77	100	12·6	2·38	1·73	7·10
					Feb. 1943		94	118	14·1	3·05	1·67	7·10
					Aug. 1943		89	101	13·3	2·73	1·60	6·00
5 and 5A	Selected	Twice daily	190	Used	Nov. 1942	Air	100	—	14·3	2·69	1·88	6·70
					Feb. 1943		88	93	14·2	2·85	1·65	6·90
					Aug. 1943		82	101	14·0	2·41	1·48	5·72
					Nov. 1942	Gas	100	—	14·6	2·83	1·90	7·20
					Feb. 1943		94	108	14·6	2·88	1·57	6·90
					Aug. 1943		89	102	14·1	2·46	1·67	6·25
8	Selected	Twice daily	165	Used	Nov. 1942	Air	93	—	14·4	2·49	1·82	6·40
					Feb. 1943		74	85	14·0	2·85	1·56	6·30
					Aug. 1943		69	96	14·4	2·50	1·25	5·22
					Nov. 1942	Gas	100	—	14·6	2·47	1·89	6·70
					Feb. 1943		94	106	14·5	2·88	1·61	6·60
					Aug. 1943		84	101	14·7	2·52	1·66	5·92

Table 15. *Flavour scores of reconstituted spray-dried milk*

Trial	Pre-heating temp. °F.	Type of pack	Stored 3½ months Time after reconstitution			6 months Time after reconstitution			12 months Time after reconstitution		
			0 hr.	½ hr.	6 hr.	0 hr.	½ hr.	6 hr.	0 hr.	½ hr.	6 hr.
1 and 1A	165	Air	Not tasted	Not tasted	+ +	Good	+	+ +	Good	+ +	+ + +
		Gas	,,	,,	+ ?	,,	+ ?	+ ?	,,	+	+
2	190	Air	,,	,,	Sl. cooked	,,	Sl. cooked	Sl. cooked	,,	Sl. cooked	Sl. cooked
		Gas	,,	,,	,,	,,	,,	,,	,,	,,	,,
5 and 5A	190	Air	,,	,,	,,	,,	,,	,,	,,	,,	,,
		Gas	,,	,,	,,	,,	,,	,,	,,	,,	,,
8	165	Air	,,	,,	+ +	,,	+	+ +	,,	+ +	+ + +
		Gas	,,	,,	+	,,	+	+	,,	+	+

+ = slight tallowiness, + + = tallowy, + + + = very tallowy.

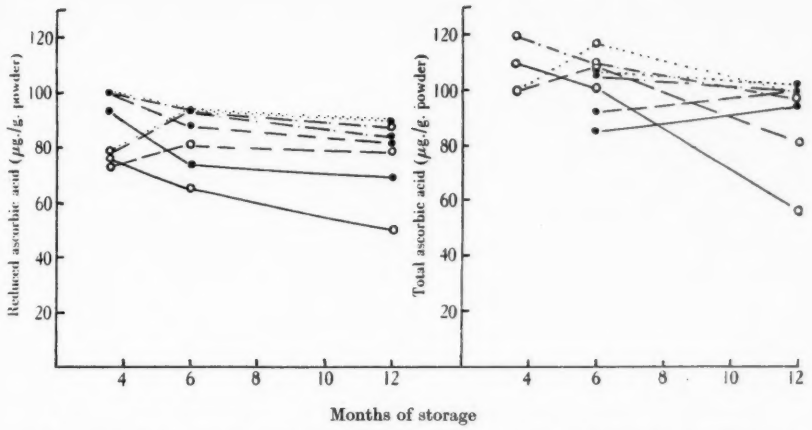

Fig. 6. Change in reduced and total ascorbic acid content of spray-dried milk powder on storage.

○ Ordinary supply. ● Selected supply.

Air pack, 165° F. ———, 190° F. — —; gas pack, 165° F. — · —, 190° F. .....

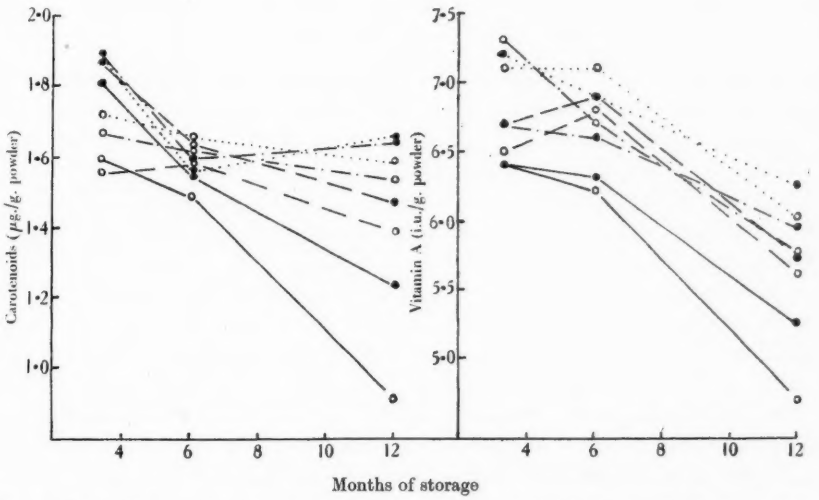

Fig. 7. Change in the carotenoid and vitamin A content of spray-dried milk powder during storage.

○ Ordinary supply. ● Selected supply.

Air pack, 165° F. ———, 190° F. — —; gas pack, 165° F., — · —, 190° F. .....

+ = slight tallowiness, + + = tallowy, + + + = very tallowy.

bination of high temperature and gas packing. It will be seen that carotenoids proved more labile than vitamin A, the loss of the former on storage varying from 42 to 7% according to treatment. The losses of vitamin A were from 27 to 12%.

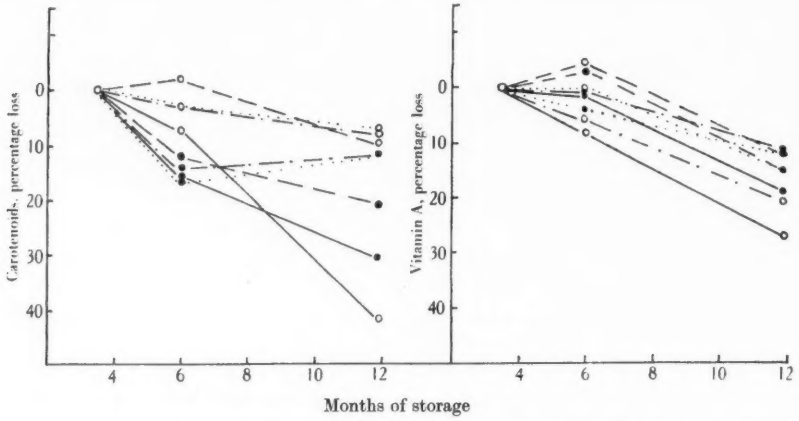

Fig. 8. The percentage loss of carotenoids and vitamin A in spray-dried milk powder on storage.

○ Ordinary supply. ● Selected supply.

Air pack, 165° F. ———, 190° F. — —; gas pack, 165° F. — · —, 190° F. .....

Stability on reconstitution of vitamin C in the various dried milks after storage for 12 *and* 24 *months*

On one occasion during the final assay of the stored powders, titrations for vitamin C of the reconstituted milks were done not only soon after reconstitution but also 1½ and 7 hr. later. It was found that a sample of milk pre-heated to 190° F. contained at first titration 89 μg. reduced ascorbic acid per gram powder, 82 after 1½ and 70 at the end of 7 hr. A powder pre-heated to 165° F. gave values of 32 and 5 μg./g. respectively. The total vitamin C content on first sampling and after 7 hr. was 102 and 88 for the 'high-temperature' sample and 96 and 13 μg./g. for the 'low-temperature' sample. It was apparent that there was rapid destruction of vitamin C in milk reconstituted from powder pre-heated to 165° F.

To confirm this observation a more elaborate experiment was planned with the collaboration of Dr E. Huntsman Mawson. Two air-packed samples were taken at random from the selected supply, one pre-heated to 165° F. and the other to 190° F. Samples were withdrawn, reconstituted and titrated immediately on opening the tins. The remainder of the reconstituted milk was stored in the dark and again sampled after ¼, ½, 1 and 4 hr. Fig. 9 shows a steady decrease in reduced ascorbic acid, amounting to 35% after 4 hr. in the 165° F. sample, but only to 6% in the 190° F. sample. The change in total ascorbic acid was less marked, the fall being 19% for the 165° F. sample and 5% for the 190° F. sample.

It is impossible to say whether these differences in stability of vitamin C after reconstitution were present from the beginning or developed on storage. The routine of titration during the tests reported in this paper was reasonably uniform and there is no

reason to suppose that comparisons between samples were vitiated by differences in rate of deterioration after reconstitution.

To determine whether the loss of vitamin C in the various milk samples begins as soon as the tin is opened and the contents exposed to air or whether it takes place after reconstitution, further experiments were carried out with powder 2 years old. Tins of milk pre-heated to 165° F. or to 190° F. were opened and the contents reconstituted immediately or tipped into a bowl, stirred to effect intimate mixing with air and allowed to stand for 1–5 hr. when they were reconstituted in the usual way. The results are given in Fig. 10. The greater stability on reconstitution was shown again by the samples pre-heated to 190° F. The losses even in air pack were small. The vitamin C of samples pre-heated to the lower temperature proved quite labile. In air pack the loss of total ascorbic acid amounted to about 50% 4 hr. after reconstitution. A corresponding gas-packed

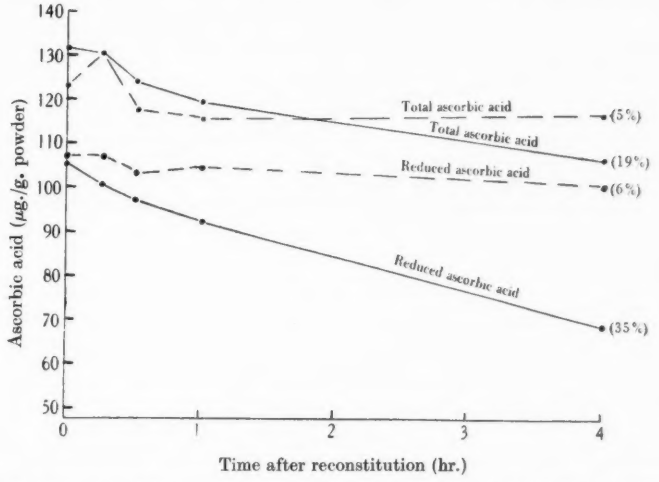

Fig. 9. The effect of pre-heating temperature on the stability of ascorbic acid in reconstituted spray-dried milk. At this test the powders were one year old. Figures in brackets denote percentage loss.

165° F. ——, 190° F. — —

sample lost less, some 16%, but even this proved less stable than the 190° F. sample in air pack. The main loss occurred in the liquid phase and the graphs show little difference in rate of loss between samples exposed to air before reconstitution and those reconstituted immediately.

Exposure of these milks to light or addition of copper effected rapid loss of the substance reacting with the indophenol reagent, showing that it was ascorbic acid and not an artefact. From the point of view of the consumer the greater stability of ascorbic acid in milk reconstituted from powder pre-heated to a higher temperature is much in favour of this treatment.

Flavour tests

These were carried out by tasting the reconstituted milk immediately after mixing, and then ½ and 6 hr. later (Table 15) as it was found that 'off' flavours rapidly developed in some milks on standing. After storage for 6 months all milks tasted immediately after reconstitution had an agreeable flavour, no difference being discernible between

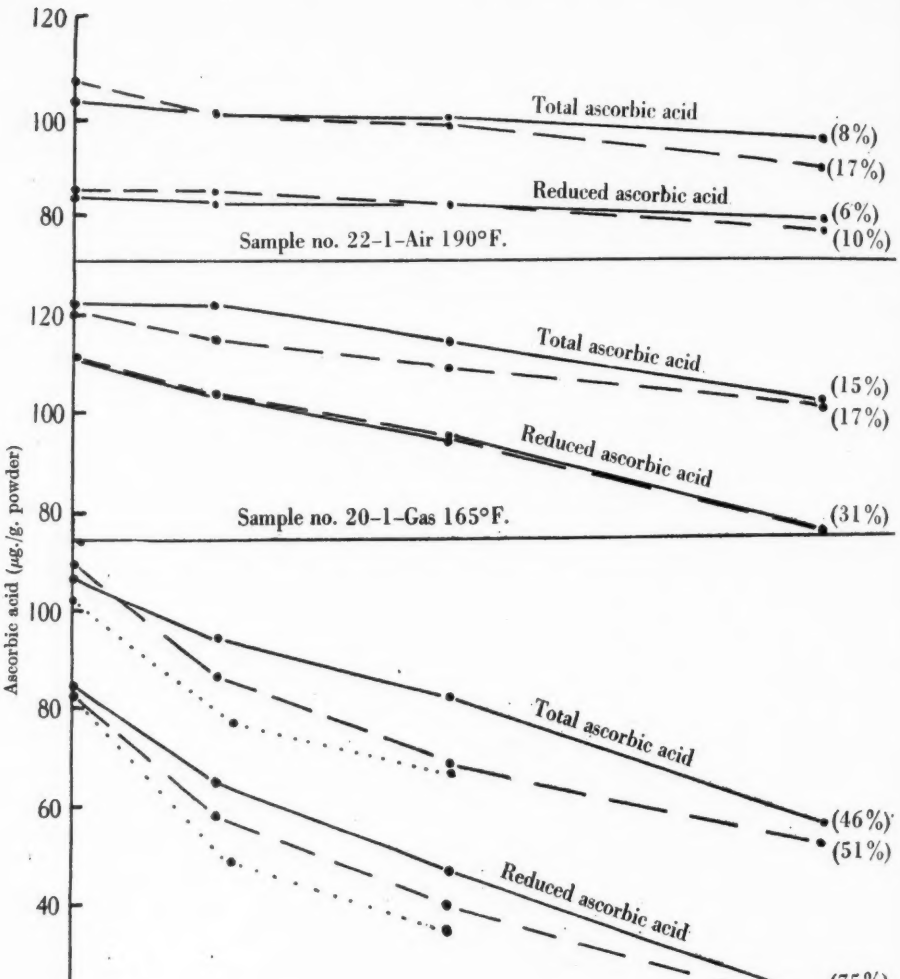

Fig. 10. The effect of pre-heating temperature and gas packing on the stability of ascorbic acid in reconstituted spray-dried milk. At this test the powders were two years old. Figures in brackets denote percentage loss.

●————● Powder reconstituted immediately on opening the tin.
●— — —● Powder reconstituted one hour after opening the tin and exposing the contents to air.
●·····● Powder reconstituted five hours after opening the tin and exposing the contents to air.

the treatments, but within half an hour the 'low-temperature' air- and gas-packed samples had developed a tallowy flavour. The flavour of the 'high-temperature' samples remained unchanged, that is to say, slightly cooked but with no trace of tallowiness. At the third test, after 1 year, the tallowy flavour was recorded in both the 'low-temperature' samples half an hour after reconstitution, but it increased further in intensity on standing. The 'high-temperature' powder was unaffected. This difference, coupled with the difference noted in the stability of ascorbic acid, would suggest that changes proceed to a certain stage in the 'low-temperature' dry powder, the second stage during which the tallowy flavour develops requiring the process of reconstitution for its initiation.

SUMMARY

The effects of the treatments described on p. 149 on vitamins A, B and C and on carotenoids and riboflavin, of the various spray-dried milks was studied in the course of a year's storage. The flavour of reconstituted milk was also examined.

There was no evidence that pre-heating to 190° F. was any more deleterious to the vitamins studied than pre-heating to the usual temperature of 165° F., and the stability on storage of vitamin A and carotenoids and of vitamin C in powders pre-heated to the higher temperature was enhanced. Gas packing afforded further protection. After reconstitution there occurred on standing in the dark in all samples of milk a certain progressive loss of vitamin C. This was much more marked in the samples pre-heated to 165° F.

Pre-heating milk to 190° F. during spray drying led to the production of a powder which was perfectly palatable even after 1 year's storage in air pack. While the taste of the milk reconstituted from powders made from milk pre-heated at 165° F. was satisfactory immediately on reconstitution, it rapidly and progressively developed 'off'-flavours on standing.

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APPENDIX

SELECTION OF RAW MILK SUPPLIES DURING THE TWICE DAILY COLLECTION PERIOD

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Many interesting features emerged from this work which will be published elsewhere, but a summary of those aspects directly related to the remainder of the experimental work may usefully be included here.

A rapid platform test seemed to be the only practicable procedure, since the experimental requirements involved (*a*) examination of milk received from *every* producer (170 in number) *every* day, and (*b*) immediate segregation of the worst milk supplies on the receiving platform.

The 10 min. resazurin test was selected as the most suitable method available, but some allowance had to be made for the fact that the milk was only a few hours old and therefore still in the lag phase on arrival. For this reason it seemed likely that only the worst supplies would be detected and that some slight reduction might occur due solely to cell content. The 10 min. rejection standard was fixed at resazurin disk 5 or less in the belief that this procedure would eliminate the worst supplies, leave a small margin for probable cell effect, and would be unlikely to divert more than 30% of incoming supplies.

Table 16

Date Aug.	Atmospheric shade temp. at 9 a.m. °F.	No. of samples tested	No. of samples rejected	% rejects	Gallonage of reject milk diverted*
10	64	168	30	17·8	1181
11	64	168	33	19·6	1232
12	62	168	30	17·8	974
13	62	170	53	31·2	1586
14	64	171	35	20·5	1243½
15	63	171	42	24·6	1438
16	67	169	32	18·9	988
17	63	168	42	25·0	1351
18	62	168	35	17·8	1228
19	60	164	35	21·3	1311
20	64	174	31	17·8	1100½
21	63	170	16	9·4	736½
22	63	175	33	18·8	1131

* This figure includes some mixed milk which was not tested but automatically diverted, but the gallonage in this category was fairly constant from day to day.

Composite samples were taken of aliquot portions from all the churns received from each producer. Only morning milk was sampled, but if this was rejected the evening milk produced on the same day was also rejected. The milk arrived in twelve loads, the average arrival time of the first being 9.39 a.m. and of the last 1.33 p.m. Samples from the last load were always under test by 2 p.m. Calculating from a milking time of 7 a.m. the samples were actually under test when from 3 to 7 hr. old. Sampling was carried out on 13 consecutive days and a total of 2204 10 min. resazurin tests and 2033 1 hr. resazurin tests was made. In addition methylene-blue reduction tests were made on a proportion of the samples, together with a few plate counts for comparative purposes. This work required a team of six workers (as a minimum).

A summary of the main results is given in Table 16, which shows that the proportion

of daily rejects varied from 9·4 to 31·2% with an average of 20%. The wide range could not be directly related to the prevailing atmospheric temperature, nor to fluctuations in the condition of churns cleansed at the creamery. It appeared to be related to some extent to harvesting operations, the proportion of rejects tending to be higher following a long day in the harvest field when the chances of milk utensils receiving adequate cleansing after the evening milking were remote on several of the farms. Of the total samples, 79·7% gave a 10 min. disk reading of 6 or 5½ and were accepted; if disk 4 or over had been taken as the rejection standard (National Milk Testing Scheme) 96·6% would have been accepted.

Of the accepted samples, only 9·9% when further submitted to the 1 hr. resazurin test gave a disk reading of 3½ or less. If the 10 min. rejection standard had been fixed at disk 4½, the samples showing a reading of 3½ or less in the 1 hr. test would have increased to 13·3%. Conversely, rejection of all samples below a 10 min. disk of 6 would have diverted 37% of the incoming supplies.

Of all samples showing a 10 min. reading of 5½ or 5, 52·9% showed a 1 hr. reading not less than 3½. Hence in many cases the quick initial reduction was due to the cell content of freshly produced milk, and possibly on bacteriological grounds some samples were wrongly condemned. At the same time, the 1 hr. tests showed that 33% of all samples showing a 10 min. reading of 5 were obviously poor bacteriologically, and acceptance of disk 5 would undoubtedly have increased the bacterial content of the bulked milk. Furthermore, the methylene-blue reduction tests showed that whilst 33% of '10 min. disk 5' samples gave a reduction time of 6 hr. and were probably satisfactory, 53% of such samples gave reduction times of 3 hr. or less. These findings support the original decision to reject at disk 5.

As the data given in Table 8 show, tests of bulked raw milk did in fact reveal pronounced reductions of plate counts and increased methylene-blue reduction times as a result of this work.

It may be of interest to note that milk from 39·4% of producers was accepted on every occasion and that a further 12·3% only failed once; under the National Milk Testing Scheme 80% of producers would have attained 'accepted' category every day.

Finally, it should be recorded that this control work involved an average delay in tipping of only 25 min., but such smooth working was partly due to special circumstances, viz. (*a*) a very spacious reception platform, (*b*) good organization of arrival times of lorries, and (*c*) adequate sampling and testing staff.

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327. SPRAY-DRIED MILK POWDER. COMMERCIAL OBSERVATIONS OVER TWO YEARS OF THE EFFECT OF HIGH-TEMPERATURE PRE-HEATING

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The beneficial effects of high-temperature pre-heating of the milk before spray drying constitute the main features which emerged from previous experimental work(1). The bacteriological advantages became obvious during the experimental period, and high-temperature pre-heating at 190°F. was introduced as standard practice following the experimental runs. Further observations have been made during two complete drying seasons, in ordinary commercial operation wherein the milk was collected once daily without special selection and pre-heated to 190°F. without preliminary clarification. This work has afforded opportunity to discover any possible practical difficulties, and also to examine a much wider range of samples than was possible during the experimental period.

1. Bacteriological features

The methods of maintaining plant cleanliness and sterility which were developed during the experiment have remained substantially unchanged, and bacteriological standards throughout the plant have, with one important proviso, been maintained at approximately the experimental level in spite of less drastic selection of raw milk supplies. As would be expected from a wider range of samples covering a 2 year period, plate counts have been obtained both higher and lower than any recorded in the experimental data, but the most important differences revealed during the post-experimental period were associated with very long drying runs. Runs up to 18 hr. duration are common during the period of maximum milk intake, but this feature did not arise during the experiment since only a portion of the factory intake was dried at that time. (In particular, during the period of twice daily milk collection two comparatively short drying runs were carried out during each 24 hr.)

The data in Table 1 show the distribution of plate counts of powder, on yeastrel milk agar, obtained from a series of 315 samples examined during the 2 yr. period. (In order to facilitate comparison with the experimental period, plate counts at 37°C. only have been considered. The powders were, however, reconstituted by dissolving direct in Ringer's solution at 50°C. in conformity with the findings of Hiscox(2), and this procedure may have yielded slightly higher counts than would have been the case if the powders had been reconstituted at 40°C. as in the experiment.) These records show that 78% of all powder samples yielded plate counts below 20,000 per g., but for runs not exceeding 8 hr. duration 86% of counts fell below 20,000 per g., 81% below 10,000 per g., and 20% below 1000 per g. The figures reveal slightly increased incidence of higher counts beyond the 4 hr. period, and a pronounced increase beyond the 8 hr. period. Detailed inspection of individual figures indicates that the critical period is reached from 10 hr. onward. The four counts exceeding 1,000,000 per g. were all obtained between 16 and 18 hr.

Such increases arise from bacterial proliferation in the evaporator and the atomizer feed tank, through which concentrated milk flows continuously at temperatures of 110

and 95° F. respectively; small quantities of milk may therefore remain dormant for several hours at the optimum temperature for growth of some organisms. This feature has been fully discussed by Crossley & Johnson (3) in connexion with milk pre-heated to temperatures not exceeding 170° F.; the work reported here has shown that the same problem arises, though much less acutely, when pre-heating is raised to 190° F. During the post-experimental period, bacteriological examinations have been made of a considerable range of plant samples taken at stages preceding spray drying. The summarized data for these samples (Table 2) present a picture similar to that of the plate counts of the powder.

Table 1. *Distribution of powder plate counts at* 37° C.

Plate count per g.		Period in run: Start–2 hr.	2–4 hr.	4–8 hr.	8–18 hr.	All samples
Less than 1,000	No.	14	19	21	—	54
	%	18·7	26·4	16·5	—	17·1
1,000– 4,900	No.	38	31	54	6	129
	%	50·7	43·0	42·5	14·6	40·9
5,000– 9,900	No.	10	7	19	2	38
	%	13·3	9·7	15·0	4·9	12·1
10,000– 19,900	No.	5	7	11	3	26
	%	6·7	9·7	8·7	7·3	8·2
20,000– 49,900	No.	8	3	11	7	29
	%	10·7	4·2	8·7	17·1	9·2
50,000– 99,000	No.	—	3	7	8	18
	%	—	4·2	5·5	19·5	5·7
100,000– 199,000	No.	—	2	1	6	9
	%	—	2·8	0·8	14·6	2·9
200,000– 499,000	No.	—	—	2	3	5
	%	—	—	1·6	7·3	1·6
500,000–1,000,000	No.	—	—	1	2	3
	%	—	—	0·8	4·9	0·9
Over 1,000,000	No.	—	—	—	4	4
	%	—	—	—	9·8	1·3
Total no.		75	72	127	41	315

Table 2. *Plate counts of plant samples on yeastrel milk agar at* 37° C. *for* 3 *days*

Period in plant run hr.	No. of trials	Pre-heater 190° F. Mean plate count per ml.	Evaporator Mean plate count per ml.	Range	Atomizer tank Mean plate count per ml.	Range
0– 2	67	360	2,800	70– 24,000	6,300	110– 99,000
2– 4	53	340	3,800	170– 44,000	7,500	190– 58,000
4– 8	49	470	26,800	160– 272,000	38,900	250– 380,000
8–18	15	320	829,000	1,100–6,000,000	1,418,000	1,300–10,000,000

Thus, during the first 4 hr. of the drying run, the plate counts of plant samples, with very few exceptions, remained at the low figures obtained during the corresponding experimental periods, and the mean figures are of the same order as those recorded in (1).

During the first 4–8 hr. of a run a definite increase in bacterial numbers was evident, but this was usually insufficient to cause a marked rise in the plate counts of the powder. In runs exceeding 8 hr. duration (particularly over 12 hr.) the increase in the bacterial population was frequently pronounced, although this did not always occur. During some individual runs, the plate counts of the concentrated milk increased from a few

hundreds to several hundred thousand per ml., resulting in considerable increases in the counts of the powder. The data in Table 2 show that the rise occurred only in the concentrated milk; it was not due to increases in the numbers of survivors in the pre-heated milk.

On three occasions breakdowns in the drying plant occurred, and opportunity was taken to record the extent of bacterial proliferation in the bulk of concentrated milk standing dormant in the atomizer feed tank. Increases in plate counts from 1600 to 48,000 per ml. in 3 hr., 7000 to 123,000 per ml. in 3 hr., and 6000 to 233,000 per ml. in 4 hr. were recorded; these figures illustrate the importance of this problem. Moreover, a portion of the flora involved may be lipolytic. Some approximate estimates of the 'lipolytic flora' have been obtained by means of plate counts on tributyrin agar, and a few examples chosen from concentrated milks sampled late in the runs may be quoted:

	Total agar count per ml. 30° C.	Lipolytic count per ml. 30° C.
1	386,000	20,600
2	293,000	105,000
3	179,000	114,000
4	63,000	4,400
5	42,000	10,500

The recent legalization of hypochlorite rinses offers a prospect of introducing a rapid sterilizing rinse during the very short 'cleaning break' of long runs, when efficient steam treatment is impossible. Some trials of this method have yielded encouraging results; thus, on one occasion the plate count of the concentrated milks was maintained below 10,000 per ml. throughout an 18 hr. run.

Coliform organisms were absent from all pre-heated milks, and present in only 6% of concentrated milks from the evaporator and in 11% from the atomizer feed tank. Coliform organisms usually occurred only in 1 ml. quantities of concentrated milk, but increases were recorded during some long runs.

2. Powder solubility

It has been shown(1) that high-temperature pre-heating of the experimental milks produced little or no adverse change in flavour, solubility, or vitamin content of the powders. Since powder manufacturers are particularly apprehensive of decreases in solubility the subsequent experience of the plant should be recorded.

Powder solubility at the plant was determined by a volume sediment method*; methods of this type are usually employed in commercial practice to facilitate rapid examination of large numbers of samples. The results were applicable only to the particular plant using this particular technique, but yielded useful comparative values. Howat, Smith, Waite & Wright(4) suggest that volume sediment methods are very sensitive to slight solubility changes in powders which are normally highly soluble, as in this particular case.

It has been shown by Crossley & Johnson(3) that solubility may be influenced by hot-air drying temperature in addition to milk pre-heating temperature. During the

* 10 g. powder were shaken 80 times with 100 ml. distilled water at room temperature. 50 ml. of reconstituted milk were centrifuged 15 min. at 1000 r.p.m. in a specially graduated tube, the upper 30 ml. decanted off and made up again to 50 ml. with water. Centrifuging was repeated for a further 15 min. and the volume of sediment read off and expressed as a 'solubility index'.

experimental trials(1) air inlet temperatures and other operating factors were maintained at constant values, and a true comparison of solubility index values was obtained between pre-heating at 165 and 190° F. The distribution of solubility values during this experimental period is shown in Table 3, series 2 and 3. Comparison of the 165 and 190° F. series shows a probably definite but insignificant loss of powder solubility in the 190° F. series; the mean value only increased from 1·23 for the 165° F. series to 1·44 for the 190° F. series.* Solubility data for the period immediately preceding the experiment are shown in Table 3, series 1. During this period pre-heating was maintained at 165° F., but hot-air inlet temperatures varied between 155 and 170° C., and the solubility figures (mean index 2·4) were actually inferior to those obtained for the 190° F. series during the

Table 3. *Summary of comparative powder solubilities*

	Numbers of samples				
Powder solubility index	Series 1 Before experiment. Pre-heating 165° F. Air inlet 155–170° C.	Series 2 During experiment. Pre-heating 165° F. Air inlet 165° C.	Series 3 During experiment. Pre-heating 190° F. Air inlet 165° C.	Series 4 After experiment. Pre-heating 190° F. Air inlet 165° C.	Series 5 After experiment. Pre-heating 190° F. Air inlet 170° C.
0 –0·5	4	1	2	6	10
0·6–1·0	2	5	4	1	9
1·1–1·5	4	1	3	9	25
1·6–2·0	3	1	4	12	43
2·1–2·5	2	2	2	2	72
2·6–3·0	6	—	1	5	120
Over 3·0	9	—	—	10	49*
Totals	30	10	16	45	328

Samples from all series

Powder solubility index	Pre-heating 165° F.		Pre-heating 190° F.	
	No.	%	No.	%
0 –0·5	5	12·5	18	4·6
0·6–1·0	7	17·5	14	3·6
1·1–1·5	5	12·5	37	9·5
1·6–2·0	4	10·0	59	15·2
2·1–2·5	4	10·0	76	19·5
2·6–3·0	6	15·0	126	32·4
Over 3·0	9	22·5	59	15·2
Totals	40		389	

* Majority occurred during a single monthly period.

experiment. Table 3, series 4, gives results secured during the remainder of the drying season immediately following the conclusion of the experiment, when operating conditions were normal, but the pre-heating temperature was maintained at 190° F. throughout. Some values for solubility index higher than for any of the 190° F. experimental samples were obtained, but there were no instances of very poor solubility.

During the following drying season (1943) a more extensive series of solubility values was obtained. The hot-air inlet temperature was maintained at 170° C. to obtain maximum output for war requirements, but this factor combined with pre-heating at 190° F. operated unfavourably on the solubility of the powder. The data for this period (Table 3,

* The solubility data recorded in (1) were determined by the method of Howat *et al.*(4), but the actual powder samples were not the same as those examined here.

series 5) show further slight deterioration of powder solubility, probably resulting from the increased air temperature.

An examination of the figures for all samples pre-heated at 165° F. and for all samples pre-heated at 190° F. indicates the extent of solubility variations which may be expected in commercial practice. The slight deterioration of powder solubility following pre-heating at 190° F. was of little practical importance, and was completely overshadowed by the marked advantages in respect of keeping quality and bacteriological quality.

3. Plant operation

Bacteriological results similar to those recorded during the experimental period have been obtained over a considerable period when an ordinary raw-milk supply of indifferent hygienic quality was used. Moreover, bacteriological results of a standard only slightly lower have been observed at a second Kestner drying plant operated by the same company, but handling a very poor milk supply. It is emphasized that such results are only possible when high-temperature pre-heating is combined with meticulous attention to plant cleaning and sterilization. Extensive arrangements have been installed for efficient steam sterilization of every portion of the plant when reassembled after cleaning, but for certain large items (e.g. holding tanks, calandria tubes, vapour separators) this method has been supplemented with advantage by additional hypochlorite treatment.

The tubular pre-heaters have operated at 190° F. without difficulty, but at this temperature deposits in the tubes are considerable. Such deposits have been removed without serious difficulty after soaking for several hours in very hot detergent of high caustic soda content; the addition of not less than 0·3% wetting agent (sodium alkyl sulphate) to the detergent reduces the soaking time considerably. The shortest possible length of pipeline between the pre-heater and evaporator has been found advantageous.

Less steam was needed in the evaporator when the high pre-heating temperature was used. The operation was thus made easier, the formation of film deposits lessened and accurate control of the specific gravity of the concentrated milk assisted.

The most serious difficulty arose during very long runs, when in spite of high-temperature pre-heating and careful plant sterilization, a minimal bacterial population during the first few hours sometimes increased later to considerable dimensions, due to multiplication in the evaporator and atomizer feed tanks. The evaporator can only be dealt with by introducing a 'cleaning break', but the time available for this may be inadequate. Recent experience suggests that a suitable hypochlorite rinse may provide a solution. Bacterial multiplication is usually greatest in the atomizer feed tank, and it has been found essential during long runs to employ two feed tanks in rotation to ensure an adequate interval for cleaning and sterilization.

It has also been found desirable to dry the concentrated milk as soon as possible after it leaves the evaporator; this has been assisted by adjusting the evaporation rate to maintain the minimum bulk of milk in the atomizer feed tank.

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328. EXPERIMENTS ON THE USE OF ANTIOXIDANTS IN SPRAY-DRIED WHOLE-MILK POWDER

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(With 3 Figures)

Waite(1) has found hydroquinone to be effective in retarding the development of tallowy flavours in spray-dried whole-milk powder when added to the milk before drying in a proportion equivalent to 0·12% of the powder. This substance, however, is known to be somewhat toxic and, at the concentration used, produced an objectionable metallic flavour in the milk. Oat flour at a concentration of 0·25% of the liquid milk afforded only slight protection, while higher concentrations imparted a noticeable oat flavour to the powder. Gray & Stone(2) have claimed that the addition of ascorbic or gluco-ascorbic acid to milk before spray drying improves the quality and keeping properties of the powder, but their data only cover a storage period of 7 weeks at room temperature. Recently, Hollender & Tracy(3) have investigated the effect of various substances as inhibitors in powder made in the laboratory on a small vacuum-roller drier. Most effective of the substances examined were hydroquinone and guaiacum resin; the latter produced a slight foreign flavour in the milk. Of moderate efficiency were ascorbic acid and sodium citrate. Sodium citrate improved the solubility of the powder, but imparted a slight salty taste. Several other substances, including oat flour, were either only feebly active or produced undesirable changes in the milk. Lea(4) has studied the antioxidant properties of a large number of substances in dry edible fats. Of the various compounds investigated the lower esters of gallic acid proved to be most satisfactory in affording marked protection against oxidation without causing discoloration or imparting foreign flavour.

The object of the present work was to investigate the antioxidant properties of some of the more promising substances when present in dried milk. In the first experiments powders were prepared on laboratory spray driers from milk to which the antioxidant had been added. The two substances which were found to be most effective, ethyl gallate and ascorbic acid, were then further studied by incorporation into milk dried on a commercial plant.

Experimental

Preparation of milk powders in the laboratory

Two small laboratory spray driers of the type described by Waite(5) were used for the testing of a number of possible antioxidants. The milk was drawn from the cow into a glass vessel, cooled, transported to the laboratory, pasteurized, pre-condensed in glass and spray dried under conditions as carefully standardized as possible. A portion of each sample of milk was used as control, and the substance under investigation added to the

remainder, usually after pre-condensation. Powders of low moisture contents of the order of 1·5% could be prepared without much variation. For higher moisture contents it was found desirable to condition the powders for some hours in an atmosphere maintained at a suitable relative humidity.

The influence of moisture content on keeping properties

A sample of milk containing 3·3% of fat was dried, conditioned to 1·9, 3·3, 4·3, 5·2 and 6·1% moisture content and stored at 47° C., 37° C. and at room temperature. Deterioration was followed by grading the reconstituted milk by a tasting panel.

Table 1. *The effect of moisture content on the colour and flavour of spray-dried milk during storage*

Moisture content %	Weeks at 47° C.						Weeks at 37° C.							
	Colour			Flavour			Colour				Flavour			
	2	4	6	2	4	6	3	6	10	16	3	6	10	16
1·9	N	N	N	ST	T	T	N	N	N	N	P	ST	T	T
3·3	N	N	N	P	ST	T, SG	N	N	N	N	P	P	P	T
4·3	SB	DB*	—	GL	GL	—	SB	B	DB*	—	SG	GL	GL	—
5·2	DB*	DB*	—	GL	GL	—	DB*	DB*	DB*	—	GL	GL	GL	—
6·1	DB*	DB*	—	GL	GL	—	DB*	DB*	DB*	—	GL	GL	GL	—

N = normal
SB = slightly brown
B = brown
DB = dark brown

P = palatable
ST = slightly tallowy
T = tallowy
SG = slightly gluey

G = gluey
GL = very gluey
* = very insoluble

Table 1 shows that at the higher temperatures there was a critical moisture content between 3·3 and 4·3% below which fat deterioration first became noticeable, and above which severe non-fatty deterioration first resulted. The non-fatty deterioration took the form of unpleasant gluey flavours, caramelization (browning) and insolubility of the protein. Within the normal range of moisture contents for milk powders it was noticeable that the sample containing 3·3% of moisture was more resistant to the development of tallowiness than that containing 1·9%, but was more susceptible to the development of stale and ultimately gluey flavours. At laboratory temperature none of the samples had developed serious spoilage when examined after 3 and 4 months, that containing 3·3% of moisture being considered best and the 6·1% sample worst.

A similar experiment carried out with milk powder containing 0·44% of ascorbic acid showed that the presence of the antioxidant retarded the development of tallowiness, particularly in the powder of lowest moisture content, but had no appreciable influence on the development of non-fatty deterioration in the powders of high moisture content. After 4 months at laboratory temperature the sample containing 3·3% of moisture was judged best, and those containing 1·9 and 4·3% next best. The sample containing 5·2% of moisture was slightly gluey and that containing 6·1% gluey and slightly discoloured.

Antioxidants

For the investigation of the effect of antioxidants the treated and untreated powders were packed in small tins or glass bottles and stored at 47° C. and/or 37° C. A gas-packed untreated powder was frequently included in the series, for comparison. After storage the reconstituted milks were graded according to the scale 0 = no 'off' flavour; 1 = a very slight 'off' flavour; 2 = a definite 'off' flavour; 3 = unpalatable; 4 = very unpalatable.

For some of the samples the rate of absorption of oxygen by the powder was also measured. Estimates of activity obtained by this means are likely to be minimal because the non-fatty constituents of milk powder absorb oxygen at a rate which may not be influenced by the antioxidant. Oxygen may also be taken up by the inhibitor and, with a readily oxidizable substance such as ascorbic acid, this may result in the treated powder absorbing oxygen more rapidly than the untreated control, though tasting tests indicate that protection is being afforded to the powder.

Ascorbic acid. Ascorbic acid at a concentration of 0·003–0·03% of the liquid milk materially lengthened the storage life of the powder without imparting any foreign flavour or colour. Data for milk containing 1·3, 2·0 and 2·8% of moisture are given in Table 2. Other experiments with powder conditioned to 3·0 ± 0·2% of moisture gave generally similar results. The degree of protection afforded by this, and by other of the antioxidants tested, seemed, however, to vary appreciably with the sample of powder.

Hydroxy-tetronic and dihydroxymaleic acids. These substances were tested because they contained the same dienol grouping (—COH=COH—) as ascorbic acid. Hydroxy-tetronic acid showed some antioxidant activity, but was considered inferior to ascorbic acid (Table 2). Samples containing dihydroxymaleic acid were, after storage, definitely inferior to the untreated control. The 'off' flavour was classed as tallowiness, but may have been due to decomposition products of the inhibitor. The rates of absorption of oxygen by the treated powders showed that these substances, like ascorbic acid, were probably being destroyed during storage of the powder.

Gallic acid and its esters. Gallic acid is freely soluble in water, but very sparingly soluble in fat. The solubility of the esters in water decreases and in fat increases as the homologous series is ascended. Tasting tests with gallic acid and with methyl, ethyl, propyl, isobutyl and amyl gallates in water and in milk showed that ethyl gallate was preferable both to the acid and to the higher esters, the latter producing an increasingly bitter or astringent aftertaste as the series was ascended. At the concentrations used (0·005–0·026% of the liquid milk) ethyl gallate had no effect on flavour.

Storage tests on powders of 1·5–1·9% moisture content showed that both gallic acid and ethyl gallate retarded the development of tallowy 'off' flavours, the figures for absorption of oxygen confirming the verdict of the tasting panel (Table 2). Occasionally with gallic acid one or more members of the panel suspected a slight 'astringent' aftertaste and, possibly for this reason, samples containing the ester were usually preferred. A test using powder conditioned to a moisture content of 3·0% gave results at 37° C. which indicated that gallic acid was a good antioxidant, though the results at 47° C. were rather erratic.

Citric acid. Tests at 47° C. with powder of 3% moisture content containing 0·003–0·017% of citric acid on a liquid milk basis showed that this substance imparted no foreign flavour to the milk, but that in the proportions used it had only a very slight antioxidant effect.

Potassium metabisulphite. This substance was tested at several concentrations and was fairly effective in retarding the development of tallowiness in the milk (Table 2). It did, however, produce a definite 'sulphurous' or boiled-milk flavour. The views of the panel on the undesirability of this flavour were rather divided. Search for a concentration which would afford useful protection without imparting any foreign flavour to the milk was unsuccessful.

Table 2. *The effect of antioxidants on the deterioration of laboratory-dried powders*

Exp. no.	Antioxidant	Conc.* %	Moisture content %	'Off' flavour score				Oxygen absorbed (mg. × 10^{-3} per g. of powder)			
	Days at storage temperature of 47°C. ...			21	34	47	61	21	34	47	61
1	Control	—	1·3	1·9	2·2	2·8	3·0	33	54	111	—
	Ascorbic acid	0·03	1·3	1·1	1·7	0·0	0·0	—	220	286	—
	Chroman	0·015	1·3	'Lubricating oil' taint				—	134	186	—
	Ascorbic + chroman	0·045	1·3	'Lubricating oil' taint				—	266	353	—
	Days at storage temperature of 47°C. ...			20	31	45	60	20	31	45	60
2	Control	—	1·3	1·1	2·2	2·8	3·1	30	48	116	219
	Gallic acid	0·02	1·5	0·7	1·0	0·9	1·6	24	40	70	83
	Gelatin hydrolysate	0·02	1·3	1·7	1·0	1·9	2·2	31	44	94	136
	Days at storage temperature of 47°C. ...			21	39	60					
3	Control	—	1·5	1·1	1·9	2·9					
	Gallic acid	0·005	1·7	0·6	1·7	2·7					
	Gallic acid	0·01	2·4	1·6	2·0	1·9					
	Days at storage temperature of 47°C. ...			21	40	63					
4	Control	—	1·9	2·5	3·2	3·4					
	Tannic acid	0·005	2·1	0·8	2·0	2·4					
	Tannic acid	0·01	1·5	1·5	1·9	1·8					
	Days at storage temperature of 47°C. ...			20	30	42	60	20	30	42	60
5	Control	—	1·7	1·5	1·8	2·4	3·3	35	45	74	202
	Potassium metabisulphite	0·005	1·7	1·2	1·7	1·0	1·8	30	58	101	218
	Creatinine	0·05	1·5	0·8	1·3	2·0	2·8	25	40	72	162
	Days at storage temperature of 47°C. ...			10	20	40	52	10	20	40	52
6	Control	—	1·4	—	1·5	2·2	3·2	17	46	108	188
	Sodium pyrophosphate	0·03	1·5	—	0·4	2·3	2·9	—	31	84	165
	Days at storage temperature of 47°C. ...			20	40			20	40		
7	Control	—	2·1	1·1	2·4	—	—	37	159		
	Sodium hypophosphite	0·03	1·5	1·2	2·4	—	—	43	279		
	Dihydroxymaleic acid	0·03	2·4	3·0	3·1	—	—	106	336		
	Days at storage temperature of 47°C. ...			20	42	62					
8	Control	—	2·0	1·9	2·4	2·9					
	Ascorbic acid	0·01	2·0	1·2	1·1	1·5					
	Potassium metabisulphite	0·002	2·0	1·2	0·9	1·9					
	Days at storage temperature of 37°C. ...			28	60	91	121	150	180		
9	Control	—	1·8	0·9	2·6	2·9	2·6	3·1	3·7		
	Hydroxytetronic acid	0·03	2·2	1·2	1·3	1·2	1·4	2·7	2·0		
	Ascorbic acid	0·03	2·8	0·0	0·9	0·5	0·0	1·1	1·0		
	Days at storage temperature of 37°C. ...			30	60	90	118	150	180		
10	Control	—	2·4	0·2	1·7	1·3	1·3	2·3	2·7		
	Ethyl gallate	0·005	1·9	0·2	0·4	0·0	0·5	0·0	0·5		
	Gallic acid	0·005	1·9	0·0	0·9	0·5	1·5	1·2	1·5		

* Based on the liquid milk. To convert to the powder basis the conversion factor is approximately 7·7 for milk of 12·7% and powder of 98% solids content.

Gelatin hydrolysate. Amino acids, especially the cyclic amino acid proline, are known to possess antioxidant properties. A neutralized acid hydrolysate of gelatin, which is comparatively rich in proline and oxy-proline, was incorporated in milk powder in a quantity corresponding to 0·02% of gelatin, calculated on a fresh milk basis. The addition had very little effect on the palatability of the milk, though a very slight 'gluey' taste was occasionally detected by one or more members of the panel in the treated sample. Both panel test and oxygen-absorption figures indicated a definite but weak antioxidant activity (Table 2).

Sodium pyrophosphate and creatinine. These substances were added with the object of neutralizing the pro-oxidant activity of traces of active copper which might be present in the milk. They produced no objectionable taste, but the protection afforded as judged both by panel and by oxygen-absorption was very small (Table 2). It is possible that these substances might have been of more value in commercial powders containing more copper than that present in the laboratory-made samples.

Sodium hypophosphite. This substance was tested as a powerful reducing agent which on oxidation would yield phosphate, itself known to possess antioxidant activity in some systems. No protective action in dried milk could be detected, though the increased absorption of oxygen by the treated powder indicated that the hypophosphite itself was probably being oxidized.

Cysteine. The thio-amino acid cysteine was just detectable in milk when the hydrochloride was dissolved at a concentration of 0·03% and the milk tasted against a control, but the treated milk tasted 'richer' and was actually the more palatable of the two. After drying to a moisture content of 3·6% and storing at 37° C. for 41 days it was found that the milk treated with cysteine had developed a very objectionable 'burnt rubber' taste which was still detectable after dilution with 10 volumes of fresh milk. The amounts of oxygen absorbed by the treated and untreated powders were approximately the same, namely 50 and 51 mg. per kg. of powder.

Tocopherol concentrates and a synthetic chroman. Two concentrates containing 20 and 40% of mixed tocopherols prepared from vegetable oils, and 6-hydroxy 22578 pentamethyl chroman, which is structurally closely related to α-tocopherol, were tested, the former at concentrations of 0·003–0·017% on the liquid milk basis in powder conditioned to a moisture content of 3%, and the latter at a concentration of 0·015% in powder containing 1·3% of moisture (Table 2). The tocopherol concentrates, which were oily substances, were dispersed in the concentrated milk by homogenization prior to drying. The chroman was dissolved in a minute quantity of alcohol. Both of these substances caused the development in milk at 47° C. of objectionable 'lubricating oil' flavours, and it was impossible to judge whether or not they possessed antioxidant properties. Production of the unpleasant flavour by the tocopherols was confirmed at 37 and at 15° C. The oxygen-absorption figures (Table 2) show that substances of this type probably undergo oxidation during storage in the powder, and the 'off' flavour may be due to decomposition products of the inhibitor.

An experiment in which the chroman and ascorbic acid were incorporated together in milk, in order to utilize the synergistic effect of the phenolic and acidic inhibitor also failed owing to the 'off' flavour produced by the chroman.

Haematoxylin. This substance, which occurs in logwood extract and can therefore be presumed to be relatively non-toxic, was tested in milk powder of 3% moisture content

at concentrations of 0·003, 0·0086 and 0·017% on the liquid milk basis. Results at both 47 and 37°C. showed that the substance had some antioxidant activity, but that it produced a definite pink changing to blue discoloration, and a 'sweetish' foreign flavour in the milk.

Conclusion. The conclusion reached from the above experiments was that, of the substances tested as antioxidants, ascorbic acid and ethyl gallate were the most promising, neither producing any 'off' flavour in the milk and both materially increasing the resistance of the powder to the development of tallowiness.

Experiment with commercial powder

Several considerations made it desirable to check the activity of these two selected antioxidants on a commercial scale. Conditions in a commercial plant, and particularly the time-temperature treatment to which the droplets of milk are subjected in a full-size drying chamber, cannot be exactly duplicated in the small laboratory machine, and as a result the laboratory-made powders sometimes appear to behave rather differently from average commercial powders, particularly in respect of the 'off' flavour they develop on storage. Moreover, some of the control powders have been found to resist the development of tallowiness for such long periods that observation of the effect of antioxidants is rendered difficult owing to the onset of various forms of non-fatty deterioration. Investigations by Mattick *et al.*(6) indicate that relatively higher preheating temperatures may account for this latter result.

Preparation of the powder. Samples of spray-dried whole milk containing ascorbic acid and ethyl gallate were prepared on a full-scale Gray-Jensen plant. Since ascorbic acid is costly and ethyl gallate cannot at present be legally added to milk powder intended for human consumption, only the smallest practicable batches of powder were prepared. To do this a 100 gal. batch of milk was pasteurized and run into a small tank from which it passed to the large spray-drier. The first quarter of the milk was dried without addition to form the control powder, and successive additions of ethyl gallate dissolved in a little water were made after the first, second and third quarters of the milk had passed into the drier. The concentrations aimed at were 0·05, 0·10 and 0·20% of the finished powder: those actually obtained in the samples of powder collected were 0·07, 0·07 and 0·20%, corresponding to about 0·009, 0·009 and 0·026% on the liquid milk basis. The divergence from the expected figures in samples 1 and 2 was undoubtedly due to the fact that in drying so small a quantity of milk as 100 gal. it was not possible to know precisely when any portion of the liquid milk entering the drier emerged again as powder; sample 1 was apparently collected a little late and sample 2 a little early.

After completion of the run with ethyl gallate, which occupied about 20 min., the drier was operated continuously for 3 hr., by which time all trace of ethyl gallate had disappeared from it. A further 100 gal. batch of milk was then dried as before, but with the addition of ascorbic acid in quantity calculated to give concentrations of 0·1, 0·15 and 0·3% in the powder. When estimated 15 days after manufacture the quantities of ascorbic acid found were: control, 0·008%; sample 1, 0·11%; sample 2, 0·14%; and sample 3, 0·31%, corresponding approximately to 0·001, 0·014, 0·018 and 0·040% on the liquid milk basis. No trace of ethyl gallate was present in any of the samples. The moisture content of the powders was estimated by drying samples in an air oven at 100°C. for 3 hr. The copper content was determined by a modification of the method of Sylvester

& Lampitt(7). The results recorded in Table 3 show that the moisture contents were all well below the critical value above which non-fatty types of deterioration develop. The copper contents suggested that very little copper contamination had occurred during the preparation of the various powders. The values for the ethyl gallate samples were slightly higher than those for the ascorbic acid samples and averaged 1·25 p.p.m. as compared with 0·7 p.p.m.

Storage tests. Samples of all the powders were stored in tins and bottles at 47° C., 37° C. and room temperature, and graded at intervals by tasting panel. The results at the two higher temperatures are given in Figs. 1 and 2. In Table 3 are collected the times required for the various powders to develop 'off' flavours of intensity 1 and 2. At a value of 1 the 'off' flavours are just beginning to become perceptible to the critical palates of the tasters; at a value of 2 the 'off' flavour would be detected by the average consumer, but the powder would still be usable.

Table 3. *Details of the ethyl gallate and ascorbic acid powders and the number of weeks required at* 47 *and at* 37° C, *for the various samples to reach 'off' flavour scores of* 1 *and* 2

Sample no.	Antioxidant* %	Moisture content %	Copper content p.p.m.	Weeks at 47° C. to reach 'off' flavour		Weeks at 37° C. to reach 'off' flavour	
				1	2	1	2
			Ethyl gallate				
Control	None	2·1	1·2	8	11	18	23
1	0·07	1·8	1·5	23	28	49	57
2	0·07	1·9	1·2	27	29	52	66
3	0·20	1·8	1·1	25	28	51	72
Mean of 1 and 2	0·07	1·9	1·3	25	28·5	50·5	61·5
			Ascorbic acid				
Control	0·008	1·8	0·8	12	14	31	34
1	0·110	1·8	0·7	15	19	48	57
2	0·143	1·9	0·6	19	20	45	57
3	0·306	1·8	0·8	20	24	45	54

* Percentages based on the powder. To convert to approximate percentages on the liquid milk basis divide by 7·7. The contents of ascorbic acid were measured 15 days after manufacture: initial values would be slightly higher.

Results. From the values given in Table 3, ethyl gallate is seen to have increased the storage life of the powder $2\frac{1}{2}$–3-fold, the lower concentration apparently being as effective as the higher. With ascorbic acid and storage at 47° C. the protection afforded increased with concentration, but the effects were much smaller than with ethyl gallate, ranging from 25 to 70%. On storage at 37° C. an increased storage life of about 60% was observed for all three concentrations of ascorbic acid.

It will be observed that there was a substantial difference between the keeping qualities of the two controls, that prepared from the same sample of milk as the ethyl gallate powders deteriorating much more rapidly than the control made from the same milk as the ascorbic acid powders. This was perhaps brought about in part by the differences in copper content between the two controls, but experiments described elsewhere(6) suggest that the main cause may have been differences in the pre-heating treatment of the liquid milk. Owing to this difference between the milks in which ethyl gallate and ascorbic acid were incorporated it is not possible to compare directly the relative merits of the two antioxidants. It would appear, however, that ethyl gallate is much the more

powerful of the two, since it extended the storage life of the shorter-lived control beyond that reached by the highest concentration of ascorbic acid with the superior control. Storage experiments at room temperature are still in progress. After 2 years, the control

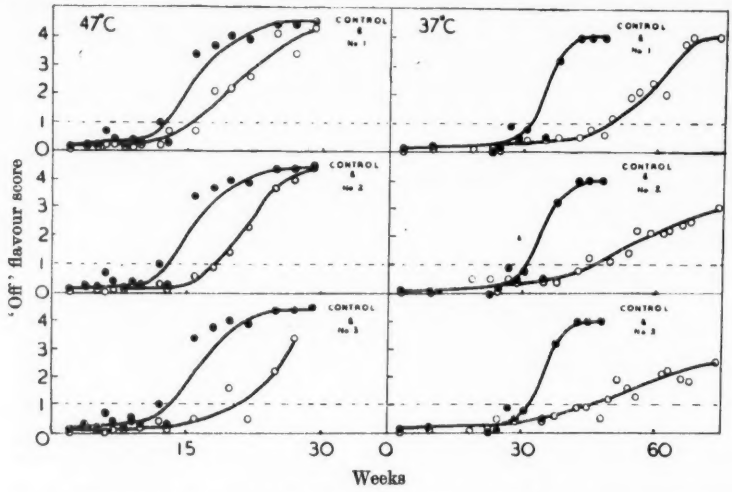

Fig. 1. Changes in flavour of the dried milks containing ascorbic acid during storage at 47 and 37° C. The initial concentrations of ascorbic acid were: control, 0·008 %; No. 1, 0·110 %; No. 2, 0·143 %; and No. 3, 0·306 %.

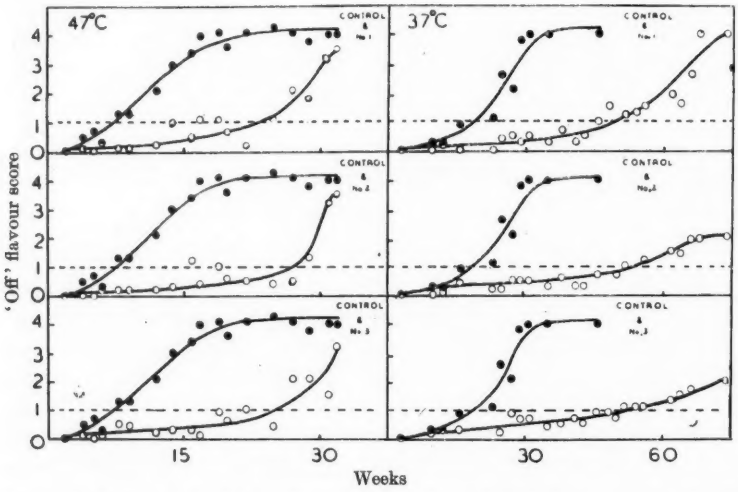

Fig. 2. Changes in flavour of the dried milks containing ethyl gallate during storage at 47 and 37° C. The initial concentrations of ethyl gallate were: control, none; Nos. 1 and 2, 0·07 %; and No. 3, 0·20 %.

powder for the ethyl gallate series was beginning to become tallowy. All the remaining samples were still very palatable.

Changes in concentration of the antioxidants during storage. Ethyl gallate was estimated by a modification of the method of Mitchell(8) and ascorbic acid by the method of

Radeff(9). The results for ethyl gallate which are summarized in Table 4 show that no detectable decrease occurred during the period of the test. Further analyses proved that even after 80 weeks at 47°C. no significant change had occurred. Fig. 3 shows, however, that the ascorbic acid concentration decreased at all three temperatures, rapidly at first,

Table 4. *The ethyl gallate content of samples during storage*

Age of powders weeks	Temperature of storage °C.	Content of ethyl gallate (%)			
		Control	1	2	3
0	—	None	0·07	0·07	0·20
9	47	None	0·07	0·07	0·22
9	37	None	0·07	0·07	0·21
9	Room	None	0·07	0·07	0·21
35	47	None	0·06	0·06	0·18
35	37	None	0·06	0·07	0·20
35	Room	None	0·06	0·06	0·20

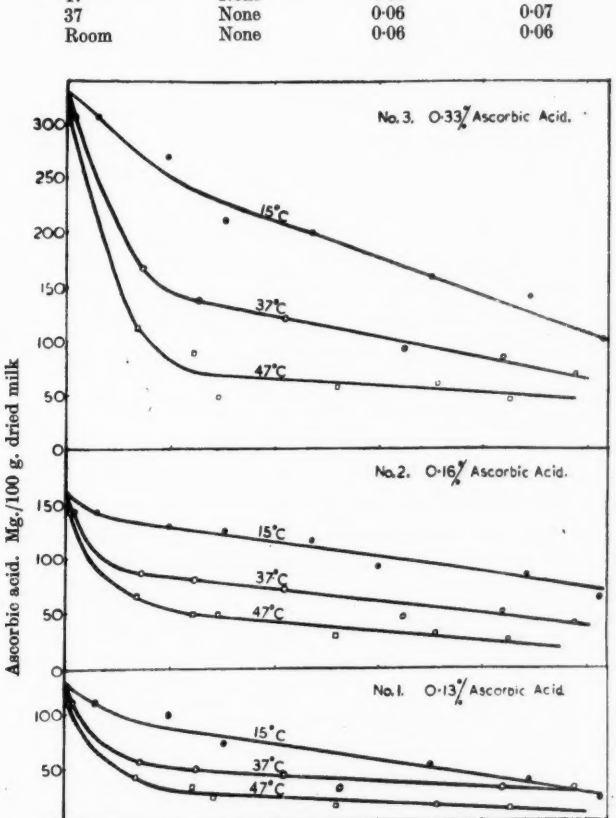

Fig. 3. The decrease in ascorbic acid during storage. Note that the ordinate scale is increased ten times for the control sample.

and then more slowly. The changes in the concentration of ascorbic acid obviously depend on at least two factors: (1) the availability of the ascorbic acid for oxidation, since the more there is present the greater will be the amount exposed and readily available, and (2) the amount of oxygen in the container. Until a certain proportion of the exposed ascorbic acid and of the available oxygen have interacted, decomposition will be relatively fast. The ascorbic acid, as measured in the powder as a whole, does not have to diminish to any one value before deterioration sets in, nor does a relatively high residual quantity of ascorbic acid prevent the powder from developing a very tallowy flavour. The stability of ethyl gallate in milk powder which has become tallowy is of interest in that antioxidants are frequently, though by no means always, destroyed while providing protection for their substrate.

The use of ascorbic acid and ethyl gallate in commercial practice. The results of these experiments suggest that ascorbic acid is not likely to be of immediate value as an antioxidant in dried milk. Its presence would be most desirable physiologically, but it would be required in quantities which would undoubtedly greatly exceed the present supply and the loss which occurs during storage would be wasteful. Ethyl gallate, on the other hand, could readily be used in commercial practice, provided the Public Health (Preservatives etc. in Food) Regulations were suitably amended. It is readily available and comparatively inexpensive. Moreover, gallic acid itself is widely distributed in nature as a constituent of tannins which are present in many vegetable foods and particularly in tea, and it is usually considered to be non-toxic. In tests carried out by Prof. J. A. Gunn and quoted by Hilditch (10) ethyl gallate was fed to or injected into mice in quantities very greatly exceeding anything which could be taken in food stabilized with this substance without any apparent ill-effects. The use of gallic acid and its esters as antioxidants has recently been patented (11).

Summary

1. Of a number of substances tested for antioxidant activity in laboratory-made spray-dried milk powder, ascorbic acid and ethyl gallate proved most promising. Both of these substances materially increased the resistance of the powder to the development of tallowiness without producing any foreign flavour in the milk.

2. The actitivy of both substances has been confirmed in factory-made spray-dried powder, ethyl gallate being much the more powerful of the two. Ethyl gallate at a concentration of 0·07% increased the storage life of powder in the accelerated tests $2\frac{1}{2}$–3-fold.

3. Ethyl gallate remained unchanged during storage of the powder, but the concentration of ascorbic acid decreased, rapidly at first and then more slowly.

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329. THE EFFECT OF THE METHOD OF RECONSTITUTION AND OF THE TEMPERATURE OF INCUBATION ON THE PLATE COUNT OF SPRAY-DRIED MILK POWDER

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Bacteriological standards are used in the assessment of the quality of dried milk powder. From a knowledge of total numbers of bacteria and the relative numbers of the various types deductions are made as to the quality of the raw milk supply, the cleanliness of the plant and the over-all efficiency of the process. Figures have been published by several workers showing plate counts of roller- and spray-dried milk powder, but the technique of reconstituting and of plating varied. Sterile water appears to have been the general reconstituting fluid, but sometimes lithium hydroxide ($N/10$ solution) was used, especially when the powder was difficult to dissolve. The temperature of the reconstituting fluid is not always mentioned, but it appears to have varied from room temperature to 60–65° C. The usual incubation temperature for the plates was 37° C., though 21–22 and 55–56° C. were also used for special purposes. In *Standard Methods for the Examination of Dairy Products*, eighth edition (1), the technique laid down mentions water, carefully warmed to 43–49° C., as the reconstituting fluid, and either 37 or 32° C. (for 48 hr.) as the incubation temperature, the choice being left to the enforcement officials having jurisdiction. American workers have for some years strongly advocated the use of 32 rather than 37° C. as the incubation temperature for routine plate counts of samples of raw milk, the advantage being that slight inaccuracies in the temperature of the incubator have less influence on the plate count which is, moreover, closer to that obtained by direct counts. The data offered in this paper show that the advantage of a similar incubation temperature (30° C. was used in these experiments) is equally obvious in the plating of dried milk powder.

During the Frome experiment (2) tins of powder were taken at each run and reserved for studies of the changes in the bacterial flora during storage. As mentioned in the report, the bulk samples were exposed to air contamination during the 20–30 min. necessary for their collection, but they were packed as quickly as possible into sterilized tins in a small room reserved for the purpose, with the minimum exposure to further contamination. It was not expected that the initial air contamination would be great, and since the moisture content of the powder was low it was not anticipated that it would seriously affect the plate count of the stored powder. The bulk powder was not mixed before distribution in the tins, but since each batch was produced from a single tank of raw milk no great difference was expected between the counts of individual tins of the same batch. This view was supported by the fact that a series of samples produced from a single tank of milk and taken from the sifter at intervals during a run showed little variation in count (Table 1).

Table 1. *Samples taken from sifter at close intervals, 23 August 1942*

Time	Moisture	Solubility*	Acidity % lactic acid	Plate count per 1 g. 37° C.	Coliform organisms	Anaerobes	Moulds
12.0 noon	—	2·0	0·90	310	Nil	Nil	Nil
12.20 p.m.	4·1	2·7	0·95				
12.25	4·2	2·1	0·86	330	Nil	Nil	Nil
12.40	3·8	2·0	0·95				
12.50	3·9	2·0	0·90	430	Nil	Nil	Nil

* Determined by a volume sediment method.

Serial tests made on tins of powder after 11–12 months' storage showed moderately even distribution in the tins when the contents were well shaken before testing, but there were differences between the plate counts of the tins. These differences were, however, usually small in comparison with differences due to other causes reported later in this paper.

Previous workers, Macy (3), Nichols (4), Crossley & Johnson (5), had reported decreases in the plate counts of spray-dried milk powder during storage, ranging from 67% in 3 months to 98·8% in 12 months. According to Supplee & Ashbaugh (6) the plate count of roller-dried milk powder decreased rapidly during the first 2 months, but was approximately constant after 2–4 months. More recent figures presented by Higginbottom (7) showed that although there was a general tendency for the plate count to decrease during storage, many samples showed no alteration in count during 6 months or even longer periods of storage. In general, the tendency to decrease in count was less marked with spray-dried than with roller-dried powder.

When the plating of the powders was resumed (after storage at room temperature for 3–4 months) a decrease in the plate counts was expected. But in fact there was a notable increase over the figures reported during the experiment—278,000 instead of 5900, and 2300 instead of 1000, for example. Increases of this order were too great to be explained by air contamination during collection or by subsequent multiplication of the bacteria in the dry powder during storage, since the moisture content was too low. It seemed more probable that the differences were due to inadvertent modifications in the technique of plating, and this was therefore studied more closely.

METHOD OF RECONSTITUTION

The technique in these preliminary tests was essentially that which had been used by Nichols(4), Crossley & Johnson(5) and Higginbottom(7), who had dealt principally with skim-milk powders. The procedure was as follows: 10 g. of powder were weighed into a sterile glass weighing bottle, added to 90 ml. of sterile ¼-strength Ringer solution at 18–22° C., contained in a wide-mouthed stoppered glass bottle and shaken fifty times with an excursion of 1 ft. in 30 sec. to dissolve. The reconstituted milk was allowed to stand for about 5 min. for the froth to subside, then 1 ml. was transferred to 9 ml. of ¼-strength Ringer solution in a test-tube and dilutions continued as for plate counts of liquid milk. Plates were poured with yeastrel milk agar and incubated at 37° C. for 3 days. The reconstituted milk was assumed to contain 0·1 g. powder per 1 ml.

With the whole-milk powder opaque globules of fat remained, and since they were of the same size, shape and appearance as many of the young colonies they added considerably to the difficulties of counting on the third day. On the fifth day the colonies were larger and only pin-point colonies were likely to be confused with the fat particles.

Table 2

Samples of powder	Plate count per 1 g. powder—yeastrel milk agar 5 days at 30° C.		
	Reconstituted at room temperature		
	Held at R.T.	Warmed to 50° C.	Reconstituted at 50° C.
Whole-milk, spray-dried, pre-heated at 165° F.	17,900	15,700	99,000
	17,000	18,600	99,000
	1,600	—	147,000
Whole-milk, spray-dried, pre-heated at 190° F.	2,300	—	6,000
	1,600	—	17,100
Whole-milk, roller-dried	1,800	—	3,900
	1,900	—	2,000
Separated milk (household milk)	1,600	1,800	9,000
	2,200	2,500	7,900

To obviate the danger of including fat globules in the count of bacterial colonies the powder was reconstituted in warm water (or Ringer solution) as in the American standard method(1), but the temperature chosen was slightly higher (50° C.). (This difficulty had already been realized at the time of the experiment and the powders were accordingly reconstituted at 40° C. during that period.) The fat globules were dispersed and the plates were clear and readily counted. In spite of the difficulty of counting, fairly consistent plate counts were obtained on reconstituting in the cold, but the figures were much lower than those obtained on reconstituting the same powder in water heated to 50° C. This was observed for various types of dried milk powder, full-cream or separated, roller- or spray-dried. Typical figures are shown in Table 2.

A full explanation for this increase has not yet been found. It was suggested that there were differences in the physical constitution of the emulsion whereby aggregates of bacteria were retained in the cold emulsion and were released only when the emulsion was made with warm water. Such aggregates, if they existed, were not broken by passing the milk reconstituted at room temperature (maintained at that temperature or warmed to 50° C.) through an emulsifier. They were not adsorbed on the fat globules, but remained in the

serum when the reconstituted milk was centrifuged. The increased count appeared to be induced only by reconstituting with hot water. Warming the cold emulsion to 50° C. did not bring the plate count to the same figure as that for the emulsion made at 50° C. This suggests that the difference in the emulsion may be due to a peculiar condition of the bacterial cells themselves as a result of the drying process. It may be that, as with spores (8, 9), the dormancy of the cells is broken by the application of heat which stimulates new growth. If this be so, then it appears that the heat shock must be applied suddenly and directly to the dry cells. Rapid or gradual heating of the cold emulsion was ineffective (see Table 3). Further study of this problem is obviously necessary.

Table 3

Plate count per 1 g. powder—yeastrel milk agar—5 days at 30° C.

Reconstituted at room temperature					
	Warmed to 50° C. in 27 sec.		Warmed to 50° C. in 13 min.		
Plated immediately	Plated immediately	Held at 50° C. for 15 min.	Plated immediately	Held at 50° C. for 15 min.	Reconstituted at 50° C. Held 15 min.
4,500	4,000	3,200	4,300	3,200	41,000

Table 4

Reconstitution temperature ° C.	Plate count per 1 g. powder—yeastrel milk agar 5 days at 30° C.	
	Powder 1	Powder 2
Room temp. (20° C.)	13,600	—
25	15,000	16,600
30	32,000	—
35	62,000	29,600
40	57,000	—
45	79,000	36,300
50	82,000	36,600
55	—	42,000
60	—	36,600

Table 5

Plate count per 1 g. powder—yeastrel milk agar—5 days at 30° C.

Reconstituted at room temperature							Reconstituted at 50° C.						
Plated immediately	Held in ice-chest			Held at 50° C.			Held in ice-chest			Held at 50° C.			Plated immediately
	1 hr.	3 hr.	5 hr.	1 hr.	3 hr.	5 hr.	2 hr.	3 hr.	5 hr.	2 hr.	3 hr.	5 hr.	
21,000	20,800	20,100	20,500	19,100	12,100	12,500	95,000	107,000	110,000	84,000	65,000	65,000	99,000
	5 hr.	12 hr.	24 hr.	1 hr.	3 hr.	7 hr.	5 hr.	12 hr.	24 hr.	1 hr.	3 hr.	7 hr.	
19,200	14,300	14,900	15,000	11,800	7,100	5,100	107,000	110,000	109,000	92,000	70,000	75,000	113,000

The choice of 50° C. as the reconstituting temperature was at first arbitrary, though it was found to agree with that shown by Howat & Wright (10) to give maximum solubility of milk powder, and is very close to that laid down in the American Standard Methods. A good emulsion was readily obtained and, since thermophilic organisms were so rare in the powder, it was thought that this temperature would not favour rapid growth of organisms during any waiting period. Later experiments with two samples of powder indicated that reconstitution at 45–55° C. gave the highest counts, and that the temperature of 50° C. had been well chosen (Table 4). Prolonged holding of the emulsions at 50° C. caused a decrease in the plate count. No tests were, however, made on powder containing large numbers of thermophilic organisms (Table 5).

TEMPERATURE OF INCUBATION

These findings were not sufficient to account for the wide differences between the original counts (the fresh powder had in fact been reconstituted at 40° C. and held at that temperature for 10 min.) and the counts of the stored powder, and (what was more disturbing still) between the counts of co-workers using the same material and ostensibly the same technique. The chief controlling factor was eventually found to be the temperature of incubation. For the tests at the National Institute for Research in Dairying an incubation room was used. This was ostensibly running at 37° C., but actually the temperature on some of the shelves was lower than this and varied by some 3 degrees from place to place. The plate counts of the milk powders varied according to the position of the plates in this incubation room. When they were placed in an incubator accurately controlled at 37° C. the counts of the stored powders were of the same order as those recorded by the other workers for the fresh powder during the experiment.

Table 6

Plate count per 1 g. of powder—yeastrel milk agar—incubated 5 days

	Tested at time of manufacture	Tested after storage at 40–70° F. for 7–8 months*							
		Incubated at					Incubating room (37° C.)		
Sample	37° C.	22° C.	26° C.	30° C.	35° C.	37° C.	Shelf A	Shelf B	Floor
20-2-A	530	17,000	24,100	24,100	17,900	1,300	—	15,000	—
22-2-G	1,430	6,500	7,700	7,300	6,800	1,800	—	4,400	—
14-2-G	720	48,000	69,000	77,000	47,000	1,300	10,000	11,000	42,000
5-1-A	328,000	3,200	8,700	8,500	2,900	730	900	1,000	3,900
A	—	58,000	94,000	101,000	69,000	1,700	3,000	39,000	63,000
B	—	9,100	18,000	18,000	11,100	700	2,100	—	7,900

* A and B had been stored for 1 month only.

N.B. Plate counts in all tables were the means of triplicate or quadruplicate platings.

Table 6 gives typical plate counts (average of quadruplicate platings) at temperatures ranging from 22 to 37° C. The most striking differences are shown by 20-2-A, 14-2-G and A, powders made from milk pre-heated at 165° F. The differences for powder from milk pre-heated at 190° F. were less marked. The highest counts (except for powders 22-2-G and 5-1-A) were obtained by incubation at 30° C. There was a sharp decrease in count between incubation temperatures of 35 and 37° C. A high proportion of the organisms growing on the plates at 30° C. either were unable to grow or grew only feebly at 37° C.

FLORA OF DRIED MILK POWDER

In methods involving incubation of plates of dried milk powders at 37° C. only, the most abundant flora of the powder may be entirely missed. It appears that these organisms are for the most part microbacteria (Orla-Jensen(11)), the maximum growth temperature of most of which is 35° C.* Their source and the true significance of their presence in large numbers in dried milk powder have not yet been

* A study of the literature suggests that the microbacteria represent a composite group of organisms highly resistant to heat and producing lactic acid in milk but also closely related to groups other than the lactic acid bacteria. The description given by Orla-Jensen(11) applies, in general, to these organisms isolated from dried milk powder, and the name 'microbacteria' is therefore retained until further study indicates the identity of the various types of organisms concerned.

determined. Although they multiply fairly rapidly in yeast dextrose broth and in litmus milk they either cause no change in reaction of the latter or only slowly produce acidity (some strains appear to induce clotting eventually). It is possible, therefore, that they may have little direct effect on the deterioration of foodstuffs of which dried milk is a major constituent, but the full effect of their enzymes on the keeping quality of dried milk products has not been studied. They have a considerable resistance to heat, apparently greater than most of the other non-sporing organisms occurring in milk, and thus tend to become the dominant flora of the powder.

A preliminary survey was made of the flora of the stored powder from the Frome experiment by picking colonies (statistical picks (12) except where all colonies on the plate could be taken) from agar plates and testing the cultures, without further purification, for growth and reaction in litmus milk, dextrose lemco broth, yeast dextrose agar stab and nutrient agar slope, catalase and Gram stain. The organisms were grouped as microbacteria, streptococci, micrococci, non-sporing and spore-bearing rods on the basis of these tests. At this stage no attempt was made at further differentiation. Thermophilic organisms were not included in this survey, but the plate counts at 55 and 63° C. of the fresh and stored powder were so low after the first 5 days of the experiment, i.e. once the evaporator had been thoroughly cleaned and sterilized, that they appear to have been negligible.

The results, which are detailed in Table 7, support the conclusion drawn at the time of the experiment from inspection of the colonies on the plates incubated at 37° C. (2) that, with improvements in the cleanliness of the plant and more particularly in the bacteriological quality of the milk supply, the relative proportion of streptococci to micrococci + miscellaneous flora was reversed. But incubation of the plates at 30° C. has revealed that even under the best conditions tried, the microbacteria far outnumbered all other types of organisms. At the lower pre-heating temperature (165° F.) they were so numerous that usually they represented 100% of the organisms picked from the plates incubated at 30° C. A comparison of the plate counts at 30 and 37° C. shows that for these powders the streptococci, though representing a high proportion of the organisms picked from the plates incubated at 37° C., were relatively so few in number that the chances that they would be included in a statistical pick of the plates incubated at 30° C. were not high. The higher pre-heating temperature, 190° F., was apparently nearer the limit of heat resistance of the microbacteria and fewer survived the heating process. There was less difference between the plate counts at the two temperatures of incubation, and the incidence of organisms other than microbacteria amongst those picked from the plates incubated at 30° C. was higher. It was assumed that the microbacteria found in the milk powder, like those described by Orla-Jensen, did not, characteristically, grow at temperatures above 35° C. There were, however, found amongst the organisms isolated from plates at 37° C. some which, though growing at this temperature, would from the preliminary survey be classed as microbacteria. These have been inserted, in brackets, in the appropriate column, but their true identity has still to be established.

Table 7 also includes a similar survey of the flora of dried milk from a Krause plant in which the milk passed in a continuous flow through the pre-heater to the evaporator, but was dried by a 'batch' process. The processes in the two plants were not exactly comparable and similar pre-heating temperatures do not necessarily imply similar heat-treatment. Milk of a much higher plate count (varying from 15,000,000 to 29,000,000)

Table 7. *Preliminary survey of colonies picked from yeastrel milk agar plates*

Table 7. *Preliminary survey of colonies picked from yeastrel milk agar plates*

Powder sample	Pre-heating temp. °F.	Plates incubated at 30°C.						Plates incubated at 37°C.					
		Plate count per 1 g. powder	Colonies picked	Types of organisms				Plate count per 1 g. powder	Colonies picked	Types of organisms			
				Micro-bacteria %	Strepto-cocci %	Micro-cocci %	Spore-bearing and other rods %			Micro-bacteria %	Strepto-cocci %	Micro-cocci %	Spore-bearing and other rods %
Ordinary milk supply:						(1) Frome experiment—Kestner plant							
28-1-G	165	255,000	24	100	—	—	—	11,200	17	—	82	18	—
29-1-G	165	71,000	16	100	—	—	—	2,500	25	(4)	48	20	32
30-1-G	190	49,000	12	92	8	—	—	600	6	—	68	—	32
1-1-G	190	106,000	18	28	72	—	—	4,200	14	—	100	—	—
3-1-G	190	6,300	12	39	38	15	8	3,400	10	—	63	37	—
4-1-G	190	68,000	14	93	—	—	7	6,800	12	—	69	12	19
6-1-G	165	500,000	16	100	—	—	—	1,300	13	—	53	7	40
7-2-G	165	71,000	16	100	—	—	—	1,800	18	(11)	33	56	11
8-2-G	165	16,900	17	100	—	—	—	1,500	14	(28)	43	7	50
9-2-G	165	520,000	14	100	—	—	—	2,100	21	(19)	66	—	34
Selected milk supply:													
12-1-G	190	4,300	12	75	25	—	—	600	6	—	50	33	17
14-1-G	165	85,000	15	100	—	—	—	2,800	14	—	38	24	38
15-2-G	165	120,000	22	100	—	—	—	2,000	20	(65)	10	16	74
17-2-G	190	8,800	14	69	—	—	31	800	8	(25)	13	37	50
19-2-G	165	20,000	20	60	—	20	20	2,000	20	(35)	5	10	85
20-1-G	165	54,000	14	71	—	7	21	3,700	12	—	7	7	86
21-1-G	190	900	12	49	8	—	43	200	4	—	—	—	100
21-2-G	190	1,600	16	76	12	—	12	300	10	—	40	—	60
						(2) Krause plant							
NIG	200	17,800	21	5	62	33	—	15,100	18	—	78	12	—
NIIG	190	14,900	24	46	37	17	—	7,300	14	—	43	50	7
NIIIG	180	64,000	17	81	—	9	10	4,700	14	—	23	77	—
NIVG	170	106,000	21	48	33	14	5	95,000	18	—	100	—	—
NVG	160	420,000	18	44	39	11	6	155,000	22	—	95	5	—

For coding see ref. (2). G = gas-packed samples.

was used in the Krause plant, but it appears that most of the bacteria were vulnerable since the plate count was greatly reduced by the pre-heating process: for example, from 154,000,000 to 660,000 by pre-heating at 160° F. Judging by the plates incubated at 37° C. it would be considered that the flora of the powder at each pre-heating temperature consisted almost entirely of streptococci and micrococci in varying proportions, a relatively few spore-bearing and other rods being present in some samples. The plates incubated at 30° C. showed, however, that significant numbers of microbacteria were also present. With a pre-heating temperature of 200° F. they were relatively few; with 180° F. they dominated the whole flora. At this temperature the difference between the plates at the two incubation temperatures was greatest.

The disparity between the plate counts of powder at incubation temperatures of 30 and 37° C. seems to be largely a function of the pre-heating temperature and period. The microbacteria, to which this disparity is presumably mainly due, are more resistant to heat than many of the other non-sporing organisms present in the raw milk. At suitable pre-heating temperatures and times their survival was high and they dominated the flora of the powder. For such powder the disparity between the plates at the two incubation temperatures was great, since these organisms were apparent only on the plates incubated at 30° C. Below this pre-heating temperature (in this experiment 180° F.) a greater proportion of the less resistant organisms survived, above it a greater number of microbacteria were destroyed, in each case resulting in a lower proportion of microbacteria in the powder flora. The rise and fall of the percentage of microbacteria with increasing pre-heating temperature is shown in the figures for the Krause plant.

With increasing severity of heat-treatment throughout the drying process, the disparity between the plate counts at 30 and 37° C. tends to disappear. The quality of the raw milk supply is another determining factor, for though no doubt microbacteria in the powder may be derived from imperfectly cleaned plants, it is probable that a more important source is the raw milk. Most samples of bulk raw milk give a higher plate count at 30 than at 37° C. and this difference is accentuated when the samples are pasteurized in the laboratory by heating at 145° F. for 30 min. The presence of microbacteria (corynebacteria—Gibson & Abdel-Malek (13)) in such samples of pasteurized milk has been demonstrated by Abdel-Malek (14).

Effect of sunlight

Another factor which was found to affect the plate count of dried milk powder was exposure of the poured plates to light. Even a short exposure to indirect sunlight caused a decrease in the plate count. In one experiment carried out in November 1943 plates were exposed in a south-south-west window from 12 noon to 3 p.m., from 12 noon

Effect of sunlight on plate count

Sample	Pre-heating temperature °F.	Plate count per 1 g. powder, yeastrel milk agar, 5 days at 30° C.	
		Unexposed	Exposed to sunlight
25-2-G	165	138,000	15,000
8-2-G	165	147,000	34,000
15-2-G	165	111,000	24,000
3-1-G	190	6,000	2,600
4-2-G	190	17,100	400
17-2-G	190	8,100	400

to 2 p.m. B.S.T. in direct sunlight. Control plates covered with metal tops were placed near the exposed plates until they were removed to an incubator at 30° C. All exposed plates showed a decrease in plate count. Experiments on other occasions showed similar results.

From the foregoing experiments it seems clear that if the aim of routine plating of dried milk powder is to estimate the numbers and types of viable bacteria in the powder, it should be reconstituted in a warm fluid. Ringer solution warmed to 50° C. was found to give satisfactory results. A warm solvent is essential with whole-milk powder if the presence of globules of fat in the plates is not to interfere with the accuracy of counting, and even with skim-milk powder, in which this difficulty does not arise, the warm solvent is preferable because it gives the higher count. To obtain as clear a picture as possible of the types of bacteria constituting the mesophilic flora, plates should be incubated not only at 37° C. but also at a lower temperature, say 30° C. In many samples of powder there are numerous organisms, e.g. microbacteria, which do not grow at 37° C., and if the lower temperature is omitted they will be overlooked. It may happen, however, that they so outnumber other organisms such as streptococci or micrococci (which grow at both temperatures) that the presence of the latter may be completely masked by incubation at 30° C. The use of both temperatures is therefore recommended. Plates should be exposed to light as little as possible before incubation.

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330. THE TECHNIQUE OF THE BACTERIOLOGICAL EXAMINATION OF DRIED MILKS

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The difficulty of reading plates prepared from roller-dried milk powders owing to their relatively low solubility was noted in a previous paper(1). Howat & Wright(2) have shown that the solubility of milk powders is greatest when reconstituted at about 50° C. In a recent investigation Hiscox(3) adopted this temperature in reconstituting spray-dried full-cream milk powders from a Kestner plant, and found that the new procedure not only increased the ease and accuracy in reading the plates but that in general it gave a higher level of counts. Hiscox further found that incubation of the plates at 30° C. (in place of 37° C. as customarily used) gave both higher total counts and a different distribution of milk flora.

The routine examination of a large number of milk powders has provided an opportunity of confirming these findings in regard to spray-dried powders and of extending the comparisons to roller-dried powders. The three variable factors investigated are (*a*) the technique of reconstitution (room temperature v. 50° C.), (*b*) the temperature of incubation (30 v. 37° C.), and (*c*) the time of incubation (3 days v. 5 days).

Experimental methods

Sampling

Approximately weekly samples of spray-dried full-cream milk were obtained in sterile 6 oz. bottles from eight plants in Great Britain during April to November 1943, the samples being taken at the point where the sifted powder leaves the plant. A few samples of spray-dried separated milk have also been examined.

Roller-dried full-cream milk, as intact tins of National Dried Milk (issued for infant feeding), was obtained by arrangement with the Ministry of Food direct from Food Offices throughout Great Britain over the period August 1943 to June 1944.

Methods of examination

10 g. of each powder were weighed out into a sterile 1 oz. wide-mouthed, screw-capped jar from which it could readily be tipped into a 12 oz. wide-mouthed straight-sided screw-cap jar containing 90 ml. quarter-strength Ringer solution. The wide-mouthed straight-sided jar has two advantages; the tendency for powder to form a cake on the shoulder of the bottle is eliminated and the use of the paper cone described by Nichols(4) and used in this laboratory in earlier investigations becomes unnecessary.

In technique A the powder was added to the Ringer solution held at room temperature (16–20° C.) and the shaking required for reconstitution standardized at 50 excursions of 1 ft. in 12 sec. Plates were prepared not less than 5 nor more than 15 min. after reconstitution.

In technique B the Ringer solution was warmed to 50° C. (by placing the jars in a water-bath at that temperature) before the addition of the powder. The degree of shaking

was moderated to 25 excursions of 1 ft. in 12 sec. and the reconstituted milk held in the water-bath at 50° C. for 15 min. After inverting the jars several times to mix, the milk was immediately plated.

Suitable dilutions in quarter-strength Ringer were plated in yeastrel milk agar (Memo/139 Foods) and incubated at 37, 30 and 55° C. Plates at each temperature were read after 3 days' incubation at 37 and 30° C. and again after 5 days. The presumptive coliform test was determined on 1 ml. reconstituted milk and for a small number of samples the keeping quality at 60° F. of the reconstituted milk was determined by the methylene-blue technique. These tests were made after reconstitution by both techniques A and B.

RESULTS

Technique of reconstitution

Reconstitution at 50° C. resulted in an increased solubility with both roller- and spray-dried powders. With spray-dried powder the fine insoluble particles disappeared and a clear agar plate resulted. Roller-dried powder prepared from full-cream milk is, of all dried milk products, the most unsatisfactory to reconstitute in the cold as the wetted powder tends to form sticky insoluble masses which adhere to pipette and jar. The extent to which this occurs varies from sample to sample. By reconstitution at 50° C. complete solution was not achieved, but the improvement was very marked and a more homogeneous suspension obtained. The fat, which held together the insoluble masses at room temperature, formed a yellow liquid on the surface of the 50° C. reconstituted milk and could be readily dispersed by inversion immediately before plating. However, some particles of insoluble powder still remained and appeared in the agar plates, but these usually resembled thin flakes and were readily distinguishable from bacterial colonies.

(*a*) *Spray-dried milk powders.* The effect of the method of reconstitution on the plate counts of spray-dried milk is shown in Table 1 in which a series of about 140 samples derived from eight different plants, is compared. For these samples, the mean plate count after 3 days' incubation at 37° C. (Table 1 (*a*)) showed over a two-fold increase by the use of technique B as compared with technique A. The mean increase varied with the plant, ranging from 20% for plant F to over 300% for plant G and was highly significant ($P < 0{\cdot}001$) for all plants except plant F ($P = 0{\cdot}20$). The increase using technique B was even more marked with the 5-day counts at 30° C. (Table 1 (*b*)), being roughly three-fold for the mean of all samples and varying for the mean counts of powders from individual plants, from 45 to over 500%. This difference was significant both for the samples from individual plants and for the combined samples from all plants.

The mean of the 3-day counts at 55° C. increased by 86% (Table 1 (*c*)): the increases again varied for individual plants, but were markedly less than for counts at 30 and 37° C. These increases were, however, significant for all plants except plant H ($P = 0{\cdot}2$–$0{\cdot}3$).

Although the *mean* counts of powder from individual plants were significantly increased after reconstitution at 50° C., the relative changes in count of individual samples derived from any one plant varied. For example, at plant G the mean increase in count at 37° C. was 327%, but this varied from 86 to 1437%; again, at plant F the variation in count was from −65% to +233%, one-third of the samples actually showing a *decrease* in count using technique B.

Re-examination of 55 of the samples of spray-dried full-cream milk after 6 months'

storage showed that the increase in the counts using technique B (Table 2) was slightly higher than that shown by the same samples when examined shortly after production.

The small number of spray-dried separated milks examined (Table 3) showed a two- to three-fold mean increase in count with technique B, i.e. the mean increase was of the

Table 1. *Spray-dried full-cream milk. Comparison of techniques A and B*

Plant	Mean log of plate count: Technique A	Technique B	Difference (B – A)	% increase or decrease using technique B	No. of samples	*t*	*P*
			(*a*) Count (3 days) at 37°C.				
A	3·59	3·80	0·21	+ 62	18	4·667	H.S.
B	3·87	4·14	0·27	+ 86	18	7·537	H.S.
C	3·34	3·67	0·33	+114	12	4·876	H.S.
D	3·72	4·25	0·53	+239	18	6·739	H.S.
E	3·60	4·02	0·42	+163	21	8·680	H.S.
F	3·74	3·82	0·08	+ 20	16	1·344	N.S.
G	4·31	4·94	0·63	+327	15	8·262	H.S.
H	2·71	3·14	0·43	+169	22	16·65	H.S.
All samples	3·58	3·94	0·36	+119	140	14·03	H.S.
			(*b*) Count (5 days) at 30°C.				
A	3·43	3·81	0·38	+140	14	4·862	H.S.
B	4·04	4·36	0·32	+109	14	9·344	H.S.
C	3·76	4·30	0·54	+247	10	8·137	H.S.
D	3·72	4·31	0·59	+287	18	6·227	H.S.
E	4·01	4·55	0·54	+247	18	3·655	H.S.
F	4·12	4·28	0·16	+ 45	11	4·752	H.S.
G	4·42	5·08	0·66	+357	15	6·454	H.S.
H	3·14	3·94	0·80	+531	20	30·42	H.S.
All samples	3·80	4·32	0·52	+231	120	20·04	H.S.
			(*c*) Count (3 days) at 55°C.				
A	3·39	3·55	0·16	+ 45	15	3·879	H.S.
B	3·23	3·46	0·23	+ 70	15	5·550	H.S.
C	2·23	2·61	0·38	+140	12	2·313	S.
D	2·30	2·67	0·37	+134	18	4·461	H.S.
E	2·41	2·66	0·25	+ 41	21	6·402	H.S.
F	2·90	3·50	0·60	+298	15	3·446	H.S.
G	2·27	2·46	0·19	+ 55	15	3·916	H.S.
H	2·57	2·63	0·06	+ 15	22	1·180	N.S.
All samples	2·65	2·92	0·27	+ 86	133	9·059	H.S.

H.S. Highly significant ($P<0·01$), i.e. chance of being alike is less than 1 in 100.
S. Significant ($P=0·05$–$0·01$). N.S. Not significant ($P>0·05$).

Table 2. *Spray-dried full-cream milk before and after storage*

	Mean log count: Technique A	Technique B	Difference (B – A)	% increase or decrease using technique B	No. of samples	*t*	*P*
Fresh 3 days 37°C.	3·76	4·16	0·40	+151	55	8·25	H.S.
6 months 37°C.	3·34	3·78	0·44	+175	55	8·95	H.S.
Fresh 5 days 30°C.	4·00	4·49	0·49	+209	55	12·81	H.S.
6 months 30°C.	3·50	4·06	0·56	+263	55	11·08	H.S.

same order as with full-cream milk powders; the increase at 55°C. was, however, not significant. Most of these samples were from plant F and in Table 4 the results for separated and full-cream spray-dried milk powders from this plant are compared. Whereas with full-cream powders, the counts at 30 and 37°C. were only slightly increased and at 55°C. very markedly increased, the reverse held for the separated milk powders.

(*b*) *Roller-dried milk powders.* Although technique B produced a more obvious improvement in the solubility of the roller-dried milks than with the spray-dried milks, the increase in count was less (Table 5). The mean counts at 37 and 30°C. showed increases of 44 and 41% respectively, compared with increases of 120 and 230% for spray-dried

Table 3. *Spray-dried separated milk. Comparison of techniques A and B*

	Mean log count						
	Technique A	Technique B	Difference (B – A)	% increase or decrease using technique B	No. of samples	*t*	*P*
3 days 37°C.	3·98	4·30	0·32	+109	14	5·689	H.S.
5 days 30°C.	4·22	4·71	0·49	+209	14	6·305	H.S.
3 days 55°C.	3·19	3·28	0·09	+ 23	13	1·592	N.S.

Table 4. *Spray-dried milk from plant F. Comparison of techniques A and B*

	Mean log count						
	Technique A	Technique B	Difference (B – A)	% increase or decrease using technique B	No. of samples	*t*	*P*
			(*a*) Separated milks				
3 days 37°C.	4·13	4·44	0·31	+104	10	4·631	H.S.
5 days 30°C.	4·34	4·73	0·39	+145	10	4·985	H.S.
3 days 55°C.	3·56	3·60	0·04	+ 10	9	1·231	N.S.
			(*b*) Full-cream milk				
3 days 37°C.	3·74	3·82	0·08	+ 20	16	1·344	N.S.
5 days 30°C.	4·12	4·28	0·16	+ 45	16	4·750	H.S.
3 days 55°C.	2·90	3·50	0·60	+298	15	3·446	H.S.

Table 5. *Roller-dried full-cream milk. Comparison of techniques A and B*

	Mean log count						
Packer	Technique A	Technique B	Difference (B – A)	% increase or decrease using technique B	No. of samples	*t*	*P*
			(*a*) Count (3 days) at 37°C.				
A	2·18	2·42	0·24	+ 74	24	3·489	H.S.
B	3·05	3·26	0·21	+ 62	29	3·758	H.S.
C	2·47	2·62	0·15	+ 41	12	4·836	H.S.
D	3·81	3·99	0·18	+ 51	17	5·806	H.S.
E	2·96	3·20	0·24	+ 74	33	5·155	H.S.
G	3·33	3·38	0·05	+ 12	32	1·041	N.S.
J	3·15	3·27	0·12	+ 32	22	1·408	N.S.
All samples	3·03	3·19	0·16	+ 44	169	2·232	S.
			(*b*) Count (5 days) at 30°C.				
A	2·31	2·62	0·31	+104	27	4·305	H.S.
B	3·14	3·37	0·23	+ 70	29	4·076	H.S.
C	3·04	3·02	−0·02	− 5	13	0·139	N.S.
D	3·73	3·95	0·32	+ 66	22	2·022	S.
E	3·09	3·25	0·16	+ 45	30	2·505	N.S.
G	3·49	3·49	0·00	0	32	0	N.S.
J	3·17	3·35	0·18	+ 51	22	2·098	S.
All samples	3·12	3·27	0·15	+ 41	175	2·354	S.
			(*c*) Count (3 days) at 55°C.				
A	2·93	2·94	0·01	+ 2	25	0·078	N.S.
B	3·49	3·63	0·14	+ 38	23	0·836	N.S.
C	1·69	1·88	0·19	+ 55	9	1·580	N.S.
D	3·26	3·38	0·12	+ 32	13	1·668	N.S.
E	2·35	2·46	0·11	+ 29	31	1·649	N.S.
G	2·65	2·73	0·08	+ 20	30	1·039	N.S.
J	2·72	2·91	0·19	+ 55	22	1·906	N.S.
All samples	2·77	2·88	0·11	+ 29	153	4·124	H.S.

milks, and an increase of 29% compared with 86% for the 55° C. count. These increases, taking the roller-dried milks as a whole, were statistically significant. When, however, the powders were grouped according to the packer (and hence the producer) the increase in count, using technique B, was not significant for any individual packer at 55° C.; at 37° C. the results for plants J and G were not significant and at 30° C. plants C, E and G failed to show a significant increase.

(*c*) *Presumptive coliform tests.* Out of a total of 184 samples of spray-dried and 260 samples of roller-dried full-cream milks positive presumptive coliform tests were obtained in only 4 and 5 samples respectively, i.e. in about 2% of samples. It was not therefore possible to determine whether the use of technique B would, in badly contaminated powders, affect the incidence of positive tests.

(*d*) *Keeping quality after reconstitution.* The keeping quality of the reconstituted milks was determined by using the methylene-blue reduction test at 60° F. The results are summarized in Table 6. Although the number of spray-dried milks examined was relatively small, there appeared to be an improved correlation ($r = -0{\cdot}63$ to $-0{\cdot}83$) when reconstitution was by technique B as compared with technique A ($r = -0{\cdot}43$ to $-0{\cdot}44$). With roller-dried milks the correlations with both techniques A and B were relatively low ($r = -0{\cdot}35$ to $-0{\cdot}40$).

Table 6. *Correlation between the keeping quality at* 60° F. *and the plate count of spray- and roller-dried full-cream milks*

Correlation between		Spray		Roller	
		No. of samples	Correlation coefficient	No. of samples	Correlation coefficient
K.Q. (M.B.R.T.) and count (3 days 37° C.)	Technique A	22	−0·43	106	−0·36
	Technique B	22	−0·83	102	−0·37
K.Q. (M.B.R.T.) and count (5 days 30° C.)	Technique A	22	−0·44	108	−0·35
	Technique B	22	−0·63	106	−0·40

(*e*) *Accuracy of replication.* In order to determine the relative accuracy obtained in replications by the two techniques, two workers each made from three to five complete replicate determinations of the plate count *on separate portions of each sample* of a small number of milk powders representative of the different methods of manufacture and drawn from different plants. The figures obtained for the count after 5 days at 30° C. are shown in Table 7. Similar results were obtained for the count after 3 days at 37° C. It will be seen from Table 7 that the coefficients of variation were, in nine out of the twelve individual workers' samples and in five out of the six meaned samples, smaller for technique B than for technique A, indicating an improved reproducibility by the former technique. The mean difference between the two workers was also smaller by technique B.

As regards results obtained on replicate counts made *on portions of the same reconstituted sample*, typical figures are given in Table 8. In seven out of the nine sets of replicates technique B again gave smaller coefficients of variation than technique A, confirming the improved reproducibility by technique B.

(*f*) *Discussion.* It is clear from the above results that the reconstitution of milk powders by holding for a 15 minute period at 50° C. provides a more satisfactory technique for the preparation of plates from both spray- and roller-dried powders. For counting purposes the plates are clearer and the difficulty of enumerating colonies as a result of

the presence of fat clumps and of particles of undissolved powder are minimized—though it should be noted that plates prepared from 1 ml. of reconstituted milk (essential with powders of low bacterial count) are still relatively opaque. Moreover, the new technique

Table 7. *Accuracy of replications obtained by techniques A and B.* (Count, 5 days at 30° C.)

			Technique A				Technique B			
Type of powder	Sample no.	Worker	No. of replicates	Mean log count	S.D.	C.V.	No. of replicates	Mean log count	S.D.	C.V.
Roller-dried full-cream milk	214/B9	H	4	3·51	0·11	3·08	5	3·48	0·19	5·38
		R	5	3·64	0·31	8·30	5	3·71	0·27	7·32
		H and R	9	3·58	0·19	5·58	10	3·60	0·25	7·03
	213/D7	H	5	3·57	0·12	3·37	4	3·81	0·20	5·17
		R	4	3·69	0·27	7·40	5	3·94	0·28	7·10
		H and R	9	3·62	0·30	8·27	9	3·88	0·24	6·23
	215/G8	H	4	2·93	0·17	5·94	4	2·99	0·11	3·57
		R	5	2·69	0·34	12·59	5	3·03	0·06	2·11
		H and R	9	2·80	0·29	10·36	9	3·01	0·07	2·47
Spray-dried full-cream milk	XA	H	5	2·98	0·14	4·55	5	3·33	0·04	1·08
		R	5	2·86	0·14	4·81	5	3·26	0·06	1·71
		H and R	10	2·92	0·11	3·92	10	3·30	0·06	1·84
	XB3	H	5	3·20	0·26	8·12	5	3·02	0·15	4·85
		R	5	2·86	0·06	2·13	5	2·87	0·12	3·45
		H and R	10	3·03	0·25	8·23	10	2·94	0·15	5·07
Spray-dried separated milk	XG	H	5	4·22	0·15	3·63	3	4·42	0·15	3·31
		R	4	4·13	0·12	2·89	5	4·45	0·09	2·05
		H and R	9	4·18	0·14	3·31	8	4·44	0·11	2·38
	XF	H and R	6	3·80	0·10	2·54	4	3·95	0·04	0·91
Mean difference between the two workers					0·17				0·11	

S.D. = standard deviation; C.V. = coefficient of variation.

Table 8. *Accuracy of replication by techniques A and B when plating out a single reconstituted milk.* (Count, 5 days at 30° C.)

		Technique A				Technique B			
Type of powder	Sample no.	No. of replicates	Mean log count	S.D.	C.V.	No. of replicates	Mean log count	S.D.	C.V.
Roller-dried full-cream milk	214/B9	17	3·39	0·20	5·85	15	3·38	0·08	2·42
	213/D7	11	3·54	0·07	2·00	17	3·77	0·07	1·90
	215/G8	10	3·40	0·16	4·76	10	3·63	0·10	2·88
	251/J7	16	3·14	0·12	3·70	15	3·21	0·14	4·45
Spray-dried full-cream milk	4G8	14	3·52	0·09	2·42	15	4·29	0·05	1·80
	4H8	19	3·46	0·08	2·25	18	3·87	0·04	1·03
	4A2	16	3·70	0·12	3·23	14	3·61	0·07	1·91
Spray-dried separated milk	4G10	18	3·76	0·09	2·33	14	4·30	0·05	1·24
	4F14	10	3·64	0·05	1·24	10	3·73	0·09	2·11

results in general in a marked increase in the plate counts, particularly with spray-dried powders, and also gives improved replicates.

The fact that roller-dried powders do not show such a marked increase in plate counts

on reconstitution at 50° C. is at first sight surprising, since at this temperature roller-dried powders show a far greater increase in solubility than do spray-dried powders. Thus Howat & Wright (2) have shown that with roller-dried powders the percentage of insoluble protein may be reduced from 50 to 70% when reconstituted at 20° C. to 20–30% when reconstituted at 50° C., whereas with spray-dried powders the equivalent reduction will be from about 5–10 to 2–5%. Indeed, with a spray-dried *separated* milk powder the increase in solubility may be equivalent to only 1–2%, although such powders (Table 3) may give 100–200% increase in count on reconstitution at the higher temperature. Such an increase is clearly not associated with a difference in either the protein solubility or the state of aggregation of the fat globules. The explanation may lie in the fact that reconstitution of spray-dried powders at 50° C. results in a change in the colloid condition of the protein suspension (as shown by a change in the opacity of the solution, which becomes 'milky' instead of 'particulate'), whereas reconstitution of roller-dried powders gives an improvement in gross solubility but fails to change the more intimate colloidal condition of the protein. Thus, with spray-dried powders the higher temperature of reconstitution may result in a breaking-up of the bacterial clumps, while with roller-dried powders the only effect will be to reduce the protein matter to a finer state of subdivision—sufficient to give a stable but 'particulate' suspension, but insufficient to release or break up the bacterial clumps. While this explanation can only be confirmed by further studies of the interrelation between the solubility and colloid condition of milk proteins and their corresponding plate counts, the main practical conclusion of the present work—as of the earlier work of Hiscox (3)—is the marked advantage obtainable by adopting reconstitution at 50° C. in the making up of milk powder suspensions for plate count purposes.

Incubation time and temperature

In order to study the effects of alterations in both time and temperature of incubation, readings of the plate count were made after 3 and 5 days' incubation at both 37 and 30° C.

(*a*) *Effect of incubation time.* The effect of the additional 2 days' incubation for dried milks reconstituted by technique B is shown in Table 9. As the differences using tech-

Table 9. *The effect of different incubation times and temperatures on the plate count of dried milks.* (*Technique B*)

Comparison of incubation for	Mean log count: 3 days	5 days	Difference (5 days – 3 days)	% increase using 5 days' incubation	No. of samples	*t*	*P*
	(*a*) Roller-dried full-cream milk						
3 and 5 days 37° C.	3·18	3·24	0·06	15	217	1·760	N.S.
3 and 5 days 30° C.	3·20	3·27	0·07	18	236	13·14	H.S.
3 days 37° C. and 5 days 30° C.	3·19	3·27	0·08	20	224	1·988	S.
	(*b*) Spray-dried full-cream milk						
3 and 5 days 37° C.	3·94	3·99	0·05	12	134	9·247	H.S.
3 and 5 days 30° C.	4·19	4·31	0·12	32	119	8·255	H.S.
3 days 37° C. and 5 days 30° C.	3·97	4·32	0·35	124	120	9·039	H.S.
	(*c*) Spray-dried separated milk						
3 and 5 days 37° C.	4·34	4·38	0·04	10	13	2·751	S.
3 and 5 days 30° C.	4·50	4·71	0·21	62	14	5·459	H.S.
3 days 37° C. and 5 days 30° C.	4·29	4·71	0·42	163	14	5·331	H.S.

nique A were of a similar order, though slightly less marked, the latter have not been tabulated.

At 37° C. the mean increase in log count, although significant for spray-dried and almost so for roller-dried milks, was not marked, corresponding to a percentage increase of only 10–15%. The mean increases for samples from individual spray-drying plants varied but slightly, i.e. from nil to 17%.

At 30° C. the mean increase in count between the 3 and 5 day incubations was greater than at 37° C. The increase was most marked with spray-dried separated milk powder (62%) and least marked with roller-dried milks (18%). With all types of dried milk the mean increase in count was, however, highly significant ($P < 0.001$). The variation in the mean increases for individual spray-drying plants was considerably larger at 30 than at 37° C. Thus plant H showed an increase of 86% whereas with plants A, B and D the increase was slight, i.e. only 10%.

(*b*) *Effect of incubation time and temperature combined.* The substitution of a 5 day incubation period at 30° C. for the usual 3 days at 37° C. resulted in moderate increase in the mean plate counts of roller-dried milks (20%), but in a marked increase in the mean

Table 10. *Spray-dried full-cream milk. Comparison of counts after incubation for 3 days at 37° C. and 5 days at 30° C. (Technique B)*

	Mean log count						
Plant	3 days 37° C.	5 days 30° C.	Difference (30 – 37° C.)	% increase using 30° C.	No. of samples	*t*	*P*
A	3·71	3·81	0·10	26	14	1·495	N.S.
B	4·31	4·36	0·05	12	14	2·298	S.
C	3·68	4·30	0·62	317	10	3·740	H.S.
D	4·25	4·31	0·06	15	18	1·081	N.S.
E	4·05	4·55	0·50	216	18	6·644	H.S.
F	3·85	4·28	0·37	134	12	4·433	H.S.
G	4·98	5·08	0·10	26	14	2·681	S.
H	3·12	3·94	0·82	561	20	5·600	H.S.
All samples	3·97	4·32	0·35	124	120	9·039	H.S.

plate counts of spray-dried milks (124–163%) (Table 9). The increases in the mean counts of spray-dried powders from individual plants showed, however, very marked variations, i.e. from 12 to over 500% (Table 10). For plants A, B, D and G the mean increases in count were not statistically significant. The first three of these plants were of similar design, but the fourth plant (G) was of the same design as plant H, which showed a most marked increase. The actual count, at both 37 and 30° C. was, however, very much higher at plant G than at plant H. This suggests that it is the working conditions of a plant rather than its specific design which are the most important factor in determining the extent of the difference between counts at 37 and 30° C.

Mattick, Hiscox, Crossley, Lea, Findlay, Smith, Thompson, Kon & Egdell(5) have published data suggesting that the pre-heating temperature of the milk before drying may influence the relation between the counts at 37 and at 30° C., the increase in count at 30° C. being more marked for powders prepared from milk pre-heated at 165 than at 190° F. Mattick *et al.* used a Kestner plant. Support for their suggestion has been obtained during the present work in an examination of milk powders produced on a Krause plant. Table 11 records the results of these tests, in which the pre-heating temperatures were varied from 160 to 200° F.—each temperature being used on 2 different days. The figures in Table 11 refer to the means for each pair of tests. The effect of increasing the

incubation period at 37° C. was insignificant. At pre-heating temperatures of or above 190° F. the count after 5 days at 30° C. did not differ markedly from that after 3 days at 37° C. At temperatures below 190° F., however, there was a marked increase in the 30° C. count as compared with the 37° C. count, though no regular correlation with temperature was evident. Although the number of samples is small, these results suggest that a marked difference in count at 37 and 30° C. may be indicative of a pre-heating temperature below 190° F., the level recommended for the preparation of spray-dried milk powders of long storage life. The small differences in count between 37 and 30° C. observed with roller-dried milks (which receive a much more severe heat treatment) may support this suggestion. After reconstitution by technique A the relation between counts at 37 and 30° C. showed a similar, but less marked, trend to that obtained after reconstitution by technique B.

(*c*) *Discussion.* In considering the most suitable choice of the time and temperature of incubation for samples of reconstituted dried milks not only the actual numerical count but also the appearance of the plate must be taken into account. At 37° C. the slight increase in colony size during the extra 2 days' incubation did not (with a few exceptions)

Table 11. *The effect of pre-heating temperature on the difference between counts at 37 and 30° C. for spray-dried milk from plant G. (Technique B)*

		Mean log count			
		5 and 3 days 37° C.		5 days 30° C. and 3 days 37° C.	
Pre-heating temperature °F.	No. of samples	Difference	% increase or decrease using 5 days	Difference	% increase or decrease using 5 days at 30° C.
160	2	−0·01	− 2	0·82	+561
170	2	0·01	+ 2	0·23	+ 70
180	2	0·07	+23	0·69	+390
190	2	0·00	0	0·04	+ 10
200	2	0·02	+ 5	0·05	+ 12

appreciably affect the ease of counting and was frequently disadvantageous, since adjacent colonies tended to become so large as to merge with one another. Moreover, with very low count powders, particularly roller-dried milks, the longer incubation time afforded further opportunity for the overgrowth of aerobic sporeformers, thus emphasizing one of the chief difficulties involved in the enumeration of counts on such powders. Although, therefore, a small and sometimes significant increase in count does tend to result from increasing the time of incubation from 3 to 5 days at 37° C., this scarcely justifies the use of the longer incubation time as a routine. At 30° C., however, the growth of many types of bacteria is appreciably slower than at 37° C. and a 5 day incubation period appears essential. Thus after 3 days some colonies were so small as to be barely detectable, while others which could be enumerated after 5 days were still invisible at 3 days by the usual counting method. An incubation period of 5 days is therefore essential with plates incubated at 30° C.

As regards the adoption of 30° C. as a routine incubation temperature, it is true that the presence of mould spores which germinate readily at this temperature may tend to interfere with the enumeration of bacteria, especially with powders which have been carelessly handled during packing. This was particularly observed in the roller-dried milk powders obtained from one packer, which regularly developed appreciable numbers

of mould colonies on plates prepared from 1 ml. of the reconstituted milk.* While this may be of use by providing an indication of the presence of mould spores without having to prepare special plates for the purpose, it will also tend to inhibit bacterial growth and thus interfere with enumeration in low count powders. With higher count powders little inconvenience will, however, be experienced, since few mould colonies develop on plates made from the higher dilutions of reconstituted milk. Since the samples of spray-dried milk powders used in this investigation were taken direct from the plant (i.e. before handling) it has not been possible to judge how far mould contamination might interfere with counts made on such powders at 30° C.

SUMMARY

1. A technique (B) for the reconstitution of both roller- and spray-dried milks is described which yields a liquid which is more satisfactory to handle, and gives clearer plates, higher counts and better replicates than the technique (A) at present recognized. The most important improvement in the new technique involves reconstitution, with a short holding period, at 50° C.

2. For spray-dried full-cream milks the mean increases in the counts obtained by technique B as compared with technique A were: for 3 days at 37° C., slightly more than a three-fold increase; for 5 days at 30° C., slightly more than a two-fold increase; and for 3 days at 55° C. an 86% increase. The corresponding increases for spray-dried separated milk were for 3 days at 37° C., a two-fold increase, for 5 days at 30° C. a three-fold increase, and for 3 days at 55° C., a 23% increase. For roller-dried full-cream milk the increases were markedly smaller, i.e. 44, 41 and 29%.

3. After 6 months' storage spray-dried full-cream milks showed a slightly greater difference between counts by technique B than by technique A.

4. A mean increase in plate count of about 10% was obtained by incubating plates for 5 instead of 3 days at 37° C. Although this increase was statistically significant, it is not considered that 5 days' incubation offers any practical advantage over 3 days' incubation.

5. At 30° C. incubation for 5 days is essential. Thus the mean count after 5 days at 30° C. showed, for spray-dried powders, at least a two-fold increase over that at 3 days, though for roller-dried powders the increase was only 20%. The percentage increase for spray-dried milks varied for individual samples and for samples from different plants.

6. There is evidence that a marked difference between the counts at 37 and 30° C. may indicate a pre-heating temperature below that recommended for the manufacture of spray-dried powders of long storage life. More information is, however, required before this supposition can be finally accepted.

7. It is suggested that, where possible, the bacterial quality of spray-dried milk powders should be determined, after reconstitution at 50° C., by the plate count at both 37° C. (3 days) and 30° C. (5 days). For roller-dried milk powders incubation at 30° C is less important.

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* The ability of moulds to grow at 30° C. may be of value since in this instance interference with mould growth ceased after its presence had been reported.

plants from which samples were obtained, and to the Ministry of Food for facilitating the supply of these samples.

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REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION A. PHYSIOLOGY OF DAIRY CATTLE

I. REPRODUCTION AND LACTATION

Reproduction

Some years ago the annual wastage of cows in dairy herds in Britain was estimated by Wright(1) to be approximately 22%, and of this about one-quarter was due to sterility. Recent figures(2) from Shropshire, Staffordshire and Warwickshire suggest that this position has not changed in the past few years. Thus in forty-eight herds containing 1925 cows there were 620 disposals in the 12 months ending September 1940. 23% of the disposals were due to sterility. The annual losses from bovine sterility in Britain have been calculated to be equivalent to £7,000,000 in milk alone. This fact is cited by Day(3) in a concise article on the control of sterility in which he discusses various factors which contribute to the problem. Some of these factors, such as contagious abortion and trichomonas infection, are pathological, but many others are functional or physiological. The present review is concerned only with the latter. Mackintosh(4), in an article on breeding and rearing to maintain the milk supply, summarizes for the practical dairy farmer many of the methods by which the incidence of functional sterility can be reduced. Both Day and Mackintosh emphasize the importance of pregnancy diagnosis, of detecting the short winter oestrus and of testing the fertility of the bull. They also mention that hormone therapy can frequently be used to great advantage for inducing oestrus. Knowledge of these factors and how best to deal with them has greatly increased during the past few years as a result of the work carried out in many research stations throughout the world.

Since the development of artificial insemination as a practical proposition, sterility in the bull has become increasingly important. A bull fertility testing service depending on the microscopical examination of semen has been established in New Zealand(5). Results for the first year involving tests of 138 bulls have been reported by Blake(6). Classification according to the laboratory results proved successful for sixty-nine of the bulls. Of these sixty-nine animals fifty-one 'good' or 'moderate' showed an average fertility rate of 57 or 58%, twelve 'unsatisfactory' bulls 38%, three 'bad' 31% and three 'sterile' 0·3%. Many of the individual results, however, were misleading. Thus one 'bad' and four 'unsatisfactory' bulls showed more than 50% fertility. The examinations in future are to include the determination of the ascorbic acid content of semen and the length of life of the sperm in recognized diluents, two factors which are believed to give good indication of the bull's fertility. The reliability of the classification in the New Zealand service would probably be greatly increased if it were possible for several samples of semen from the same bull to be examined over a period. The need for examining a number of samples has been stressed by Anderson(7) and also by Swanson & Herman(8). The latter authors recommend that at least five samples should be collected from the same bull over a period of a fortnight. Anderson(9) has discussed in detail his methods of examining and evaluating semen. He finds that the proportion of abnormal spermatozoa is about 10% for normal and 25% for abnormal bulls, and points out that since the bulk of the ejaculate is composed of accessory secretions, its volume indicates the activity of the accessory glands rather than of the testes. He was unable to determine any seasonal influence on sperm quality and found no evidence to suggest that sperm quality was affected by the frequency of the collection or by the length of the interval since the previous collection. Other recent publications by Anderson(10, 11) deal with the clinical significance of the *p*H of bull semen and with further investigations on the semen of fertile and sterile bulls. Routine laboratory examination of 645 semen samples has been made by Dougherty & Ewalt(12). The data they record includes the volume of ejaculate, degree of motility, *p*H, spermatozoa counts and details of the numbers and kinds of abnormal spermatozoa. These authors found little correlation between their

laboratory findings and breeding efficiency, semen described as excellent, good, fair and poor giving 53, 41, 47 and 50% pregnancies respectively. The more motile the sperm, the more rapidly the *p*H changed after the sample was collected. Herman & Swanson(13) have published a valuable bulletin in which they have reviewed the literature on the morphology of spermatozoa and breeding record, and in which they record the examination of 342 ejaculates from fifty-five bulls (Jerseys, Guernseys and Holsteins). In this and in other work(8) these authors found that volume, density, initial motility, percentage of abnormal types and *p*H could not be correlated with fertility. Much variation was found both within and between bulls. The best index of semen fertility was found to be the time for which the sperm survived with good motility at 40° F. For separate ejaculates from the same bull initial motility was roughly correlated with viability on storage. Phillips, Lardy, Heizer & Rupel(14) also found that a fairly reliable estimate of a bull's potency may be obtained from the time of survival of the sperm, but in their view the ascorbic acid content of the semen and blood plasma of the bull is also very important. They found that of twenty-nine bulls showing sexual indifference, inferior semen or poor breeding record, only four failed to respond to ascorbic acid treatment. The average ascorbic acid content of the semen of good breeders was 6·2 mg./100 ml. Values below 2 mg. were found for poor breeders and values greater than 8 mg. for bulls with questionable or erratic records. For those with low values the average dose recommended is 8 mg. ascorbic acid injected subcutaneously. Phillips, Lardy, Boyer & Werner(15) have concluded that ascorbic acid can also be used successfully in treating certain types of sterility in the cow. Excluding cows with anatomical abnormalities, 60% of the 'hard to settle' cows responded to ascorbic acid treatment. The use of ascorbic acid therapy with special reference to the work of Phillips and his colleagues has been reviewed recently by Garcia-Mata(16). Directions for treatment are given by McIntosh(17) and by Miller(18).

Chemical studies of bull semen have been made by Davis & Williams(19) and by Zittle & O'Dell(20). The latter authors have fractionated spermatozoa by disintegration with supersonic waves and separation of the parts by fractional centrifugation. The tail contained most of the lipoid, the head most of the phosphorus and nucleic acid. The values for sulphur, cystine and nitrogen were similar for all fractions. Lardy & Phillips(21) have shown that spermatozoa contain a succinic dehydrogenase and an alkaline phosphatase. The latter is also present in the seminal fluid. Both sperm and fluid contain riboflavin. By studying the effect of certain inhibitors and activators of sperm metabolism, the same authors(22) were able to suggest that the sperm obtain their energy preferentially by glycolysis, but that if glycolysis is inhibited they obtain it from the phosphatide. The effect on sperm metabolism of gramicidin, a bactericidal agent for Gram-positive organisms, has been studied by Henle & Zittle(23). By thyroidectomy experiments Petersen, Spielman, Pomeroy & Boyd(24) have found that thyroid deficiency in the bull has no apparent effect on spermatogenesis or the normality of the sperm, but that it completely inhibits sexual desire. Normal sexual behaviour was restored by desiccated thyroid and dinitrophenol, both of which increase metabolic rate. It was partially restored by testosterone propionate, but benzidrene sulphate was ineffective.

The importance of being able to detect oestrus in cows if their reproductive efficiency is to be maintained at its maximum is well known. Scott Blair *et al.*(25, 26, 27) have studied the rheological properties of bovine cervical excretions and have described an oestroscope which they have used for this purpose and which measures the flow-elasticity (p) of the cervical mucus. For forty-one samples from cows in or near oestrus p averages 14·7 mm. ± 9·6 as compared with 2·4 mm. ± 1·4 for 143 samples from cows in mid-cycle. When p is greater than 5 mm. the cows are probably in or very near oestrus. The authors point out that an instrument such as this would be particularly useful for stock-breeders, especially for predicting oestrus in animals in which it is liable to be missed because the external signs are weak or because oestrus occurs at night. The same authors found that the total nitrogen, dry matter and viscosity of the secretions reach minimum values at or near the time of oestrus, the variation in the total nitrogen being great enough to serve as a test for oestrus.

The use of hormones for inducing oestrus is becoming increasingly important. Bottomley, Folley & Watson(28) have found that of nine cows which failed to show signs of oestrus, four responded to intravenous injections of Antuitrin S (a pregnancy urine extract) and three to

intramuscular injections. Treatment with this preparation at the time of service increases the fertility of cows which show regular oestrus but which do not conceive probably owing to failure to ovulate or to deficiency of progesterone following ovulation. (The fertility of three bulls was also restored by injections of Antuitrin S.) In a short article on the induction of oestrus by treatment of persistent corpus luteum and by hormone therapy Bhattacharya & Hammond(29) recommend the subcutaneous injection of an extract of pregnant mare serum when no corpus luteum can be detected in the ovaries. Folley & Malpress(30), in a later article, quote results which show that the use of oestradiol dipropionate or monobenzoate has several advantages over the pregnant mare serum. It is effective in anoestrus conditions even when these conditions are due to the presence of corpus luteum. There is no danger of superovulation. One intramuscular injection is sufficient, and the oestrogen is cheaper and more readily obtained than pregnant mare serum. These findings have been confirmed by Anderson(31). The use of stilboestrol dipropionate for inducing oestrus in cattle has been discussed briefly by Brownlee(32), Gould(33), Stuart(34), Anderson & Bugg(35) and Allen(36). In the report of a discussion on the subject(37) emphasis is laid on the danger of indiscriminate use of stilboestrol dipropionate and the advisability of clinical examination before the oestrogen is applied. Liess(38) has described the successful induction of oestrus by administering 'sexocretin', which is a solution of di(*p*-hydroxyphenyl) hexene in oil. The author claims that even when this substance is given orally oestrus occurs within 24 hr., and that it occurs in cows with healthy genitalia quite independently of the oestrus cycle, in cows 6–9 months pregnant and in castrated and sexually immature cows. Pregnancy is not interrupted and lactation is only very slightly inhibited. The author recommends that service should be deferred until the second oestrus after treatment, since it is improbable that ovulation follows the oestrus induced by sexocretin. A most informative article dealing with many aspects of sterility, including hormone therapy, has been published recently by Runge(39).

The sexual cycle and ovulation in the cow have been investigated by Hvatov(40) and by Brewster, May & Cole(41). Brewster and his co-workers found that the time of fifty-three ovulations detected by rectal palpation ranged from 2 hr. before to 26 hr. after oestrus with an average of 13 hr. after. The minimum time taken for sperm to reach the upper third of the Fallopian tube was 5½ hr. for mature cows and 4½ hr. for heifers. Experiments with dead sperm gave no indication that sperm travel is influenced by uterine contractions.

Menstruation frequency and its relation to conception has been investigated by Trimberger(42), who finds that of 400 cows only 303 showed evidences of menstruation, and that 225 of these showed menstrual discharge on the second day following oestrus. Whether the animals were served or whether they conceived after service did not appear to be related to the occurrence of menstruation as judged from external evidence.

The *p*H of various fluids of the genital tract of the cow have been studied by Lardy, Pounden & Phillips(43), who found average values of 6·4 (6·0–6·7) for anoestrus vaginal douche, 8·3 (7·6–8·9) for cervical fluid, 6·8 (6·6–7·15) for uterine wash, 7·12 (7·0–7·4) for amniotic fluid and 7·6 (7·5–7·7) for follicular fluid. The authors concluded that vaginal douches for sex control by *p*H modification are not likely to be effective. The subject has also been investigated by Smith & Asdell(44). The chemistry of the blood during the oestrus cycle has been studied by Eveleth, Millen & McNutt(45) who found that blood phosphorus decreased and blood glucose increased during oestrus while serum calcium and magnesium remained unaffected.

The early diagnosis of pregnancy is of great importance for the maintenance of reproductive efficiency in cattle. So far the most reliable method appears to be direct physical examination as carried out by the trained expert who can often diagnose pregnancy at the end of 5 or 6 weeks and with certainty after 9 weeks. The method of making the diagnosis is discussed very fully in a most informative article by Gould, Hignett & Steele-Bodger(46). A technique for the examination of the genital organs and a basis for making a prognosis have also been published by Fincher(47) and by Murray(48). In the past, many workers have tried to devise a test by which pregnancy could be ascertained at a much earlier date than by physical examination, but so far the results have not been successful. A method depending on the effect of the milk of the cow on the crystallization of copper chloride has been described by Sagorsky(49), but apparently the test at present only detects a change after pregnancy has continued for 4 months.

A promising approach has been opened up by the experiments of Scott Blair, Cowie & Coppen(50) on the rheological properties of bovine cervical secretion. They have observed a marked difference in the slope of the flow curves of the secretion from non-pregnant cows as compared with cows which were 3 months pregnant. Further investigations are in progress to find whether this difference is likely to be of value in diagnosing pregnancy at an early date.

The effect of age on reproduction has been studied by Bowling, Putnam & Ross(51), who made a statistical analysis of the number of services required for conception in their institute herd during 1920–38. In all 706 cows and forty-three bulls were involved. When age was not considered, an average of 2·8 services were required for the first conception as compared with 1·8 for subsequent conceptions. Breeding efficiency decreased with the age of the bull, but did not become significant till after an average age of 6 years. Bulls of 13 years old were significantly less efficient than bulls of 6 years old. Records published by Mentzel(52) for 8000 cows in thirty-nine herds over a period of 14 years show that for these herds sterility was relatively slight up to 6 years of age, but that it then increased up to 20% at 11 years.

The importance of inheritance for good fertility is stressed in a bulletin from Oregon by Jones, Dougherty & Haag(53). Tutt(54) and Smythe(55) have discussed the problem of sterility in the Guernsey breed. Smythe points out that constant selective breeding to increase milk production may well be developing undesirable factors in the reproductive efficiency of the breed.

Obligatory control of the reproduction in cattle has been introduced in Thuringia(56). It is claimed(57) that the scheme has resulted in an annual increase in the rate of calving from 68 to 73%, which is equivalent in Thuringia to an additional 11,000 calves per annum.

For a method of determining the reproductive efficiency of cattle reference should be made to a paper by Kingman & Kingman(58), who present a method of keeping records and show how to arrange the breeding history of individuals so that either groups or individuals can be studied separately or collectively. They also publish tables supplemented by charts and graphs for statistical interpretation.

Sterility in sheep has been most lucidly dealt with in an article by Hammond(59). He stresses the importance of feeding the ewes particularly well during the last month of pregnancy and refers to recent experiments in which hormone therapy enabled the breeding season to be advanced and two lamb crops to be obtained within a year. Details of the experiments with hormones are given by Parkes & Hammond(60, 61). Other hormone experiments with ewes have been reported by Polovceva & Fomenko(62).

It is frequently believed that the number of bull calves born annually significantly exceeds the number of heifers. No proof of this assertion has been obtained in recent years. Thus Gowen(63) reports that in 3559 births 50·5% of the calves were male. There was no indication of any difference between sires or between dams in their tendency to affect the sex ratio, nor did the sex ratio appear to be affected by the age of the parents.

A very detailed study of the occurrence of twins and triplets in cattle has been made by Belic(64). The work is too comprehensive for adequate treatment in the present review. Examination of statistics showed that of 2,335,208 births 1·3% or 30,499 were twins, the percentage given by various authors varying from 0·3 to 29%. Of the total number of twin births 5% were monozygotic. Erdheim(65) has found that in dairy cattle one pair of twins occurred in every eighty-nine pregnancies, and in beef cattle one in every 126. Of seventeen twin pregnancies in the dairy animals two were unilateral. Of nineteen pairs of twins in the beef breeds three were unilateral. Early recognition of the free-martin condition in heifers twin born with bulls is described by Swett, Matthews & Graves(66). The characters which are considered useful in making the diagnosis are a retarded or a typical development of the udder and an enlarged clitoris or a 'rudimentary penis' taking the form of a skin fold extending part or all the way from the navel to a point above the rear attachment of the udder. Exceptional instances of fertility in cows are recorded by Tarr(67) (quintuplets) and by Schmidinger(68) (eleven calves in 4 years).

Lactation

(1) *Hormones*

The development of the mammary gland

The fact is now well established that in intact animals oestrogens and progesterone stimulate the growth of mammary tissue. In 1937 Gomez & Turner(69) found that this stimulation did not occur in the hypophysectomized animal and therefore suggested that the ovarian hormones might produce their action by stimulating the secretion of mammogenic hormones in the anterior pituitary. Later Lewis & Turner(70) found that a fat-soluble extract of the anterior pituitary stimulated the growth of the duct system, but not of the lobule-alveolar tissue. This fat-soluble factor they termed Mammogen I. Later, Mixner, Lewis & Turner(71) found that when the fat-soluble extract was injected into fifty-six young spayed mice during 6–30 days only two showed any growth of lobule-alveolar tissue, but that when fresh anterior pituitary was given during 4–8 days to 114 similar mice, lobule-alveolar development occurred in fifty-seven of the animals. The authors concluded from this and other experiments that lobule-alveolar growth, as distinct from duct growth, is stimulated by a pituitary factor which is insoluble in ether and which they named Mammogen II. It was possible that in some of the experiments the stimulating properties attributed to Mammogen I and Mammogen II might have been caused by the presence of progesterone, but Trentin, Mixner & Lewis(72) tested the anterior pituitary extracts for progesterone by the sensitive technique of McGinty, Anderson & McCullough(73) and found none. The assay of Mammogen II has been described by Mixner & Turner(74), who define the mouse unit of Mammogen II as the amount of material required to be injected daily for 10 days to secure definite lobule-alveolar development in $50 \pm 10\%$ of ten or more ovariectomized nulliparous mice weighing between 12 and 18 g. Photographs show the amount of growth regarded as a minimum response. The female mice used for the assay are ovariectomized 4 or 5 days before treatment. A mammary gland is removed and stained for examination, and only animals with smooth duct systems are used for the test. Other substances which stimulate the pituitary to secrete Mammogen II may also be assayed in this way. Thus, increasing amounts of progesterone, together with a constant amount of oestrone, were found to give good graded response. The synergism which exists between oestrone and progesterone was demonstrated by the same authors(75) using this technique. They found that with the dose of progesterone fixed at 1 mg., optimal response to the lobule-alveolar test was obtained when oestrone was given in amounts ranging from 40 to 133 i.u. 6 mg. progesterone were required when given alone to produce the same results. A similar synergism was found between the anterior pituitary lobule-alveolar factor itself (Mammogen II) and oestrogens. Thus, 35 mg. of fresh pituitary tissue with 75 i.u. of oestrogen were equivalent to 400 mg. of the same pituitary tissue administered alone. Pregneninolone(76) was about half as active as progesterone, and its activity was increased five times in the presence of oestrogen. Several other substances were also compared with progesterone by the same technique(77). The following substances induced lobule-alveolar growth: desoxycorticosterone acetate and dehydroandrosterone (about 33% of the activity of progesterone), diethylstilboestrol (about 25%), Δ^5-3-hydroxy-21-acetoxypregnene-20-one (about 6%) and methyl testosterone (about 4%). Testosterone and its propionate were inactive. The synergism between oestrone and progesterone has also been studied by Lyons & McGinty(78) and by Scharf & Lyons(79) using immature male rabbits. When 120 i.u. of oestrone were given along with 0·25, 1·0, 4·0 and 8·0 i.u. of progesterone, 1 i.u. of progesterone gave the best synergism. When in the second series of experiments the amount of oestrone varied from 30 to 960 i.u. the greatest proliferation of mammary tissue resulted from 1 i.u. of progesterone plus 240 or 960 i.u. of oestrone. The results of these experiments are well illustrated by photomicrographs.

As further evidence for the existence of pituitary mammogenic hormones Mixner & Turner(80) have published results of experiments in which the lobule-alveolar development in ovariectomized virgin mice kept at 35°C. was studied. It was found that the response to progesterone and oestrone was greatly reduced at 35°C., whereas, when a pituitary extract containing lobule-alveolar factor was injected, mammary development was immediately stimulated. The authors

conclude that, as in previous work which they cite, the secretion of hormones by the pituitary is readily influenced by such factors as temperature and inanition (81), but that the growth-promoting action of these hormones on the tissue which they stimulate is not so affected. The oestrogens therefore had little influence at 35° C. since they had to act by way of the pituitary, whereas the activity of the pituitary extract itself was unimpaired. Further evidence supporting the theory that mammogenic hormones are secreted by the anterior pituitary has been published by Mixner, Bergman & Turner (82) from experiments with mice, by Gomez (83, 84) with guinea-pigs and by Reece & Leonard (85, 86), Cary (87) and Lewis, Gomez & Turner (88) with castrated male and hypophysectomized female rats. In the last of these experiments extracts of pituitaries from pregnant cattle caused mammary development, but did not cause any statistically significant increase in the weights of the thyroids, ovaries, uteri and adrenals of the injected animals as compared with those of untreated controls. The suggestion is made that the pituitary mammogenic factors do not act through any of these organs. Subsequent experiments by Trentin, Lewis, Bergman & Turner (89) showed that on fractionating pituitary extracts most of the mammogenic activity accompanied the protein material. They also showed that since castrated male mice responded to pituitary extracts, the mammary growth could not be ascribed to gonadotropic hormones. Further confirmation of the importance of the pituitary in mammary development has been published by Leonard & Reece (90), who were unable to cause mammary growth in hypophysectomized rats with oestradiol propionate, testosterone propionate and desoxycorticosterone acetate either alone or in various combinations even in animals whose body weights increased. Local application of oestradiol was also ineffective in causing mammary development in the absence of the pituitary.

Most of the evidence in favour of Turner's theory that specific mammogenic hormones are secreted by the anterior pituitary has been published by Turner and his colleagues from Missouri. The theory, however, is not universally accepted. For example, Nelson (91), continuing his earlier work of a similar nature, has shown that when oestrogens are applied externally to one mammary gland of gonadectomized male and female guinea-pigs, mammary growth occurs only in the treated gland, but if in stimulating mammary development oestrogens act solely by causing the pituitary to secrete mammogenic hormones into the blood, all the mammary glands would be expected to show a similar response. The local development of mammary glands treated with oestrogens has also been demonstrated by Speert (92) for immature male rhesus monkeys, by Lewis & Turner (93) for male rabbits and by Gardner & Chamberlin (94) for male mice. Fredrikson (95) has found that oestradiol monobenzoate with progesterone causes growth of mammary ducts and alveoli in ovariectomized hypophysectomized rabbits. Mammary development also proceeded in rabbits which were hypophysectomized soon after the induction of pseudo-pregnancy in which the corpus lutea were maintained by oestradiol monobenzoate. Chamorro (96, 97, 98), whose work has recently been extended to experiments involving pituitary and oestrogen implants on rats (99), has also observed normal mammary growth in hypophysectomized female mice treated with gonadotrophins to maintain ovarian function in spite of the absence of the pituitary. His experiments showed that simultaneous injection of desoxycorticosterone acetate and oestradiol benzoate stimulated mammary development, though separately these substances were ineffective. Desoxycorticosterone in the presence of oestradiol propionate has also been found by Gardner (100) to cause marked mammary development in hypophysectomized male mice. Its importance in the mammary growth of the rhesus monkey has been studied by Speert (101). In experiments by Gardner & White (102) mammary development did not occur when prolactin was injected into male mice unless oestrogens were also given. In further similar experiments (103) proliferation of the ducts was obtained by administering oestrogen in combination with progesterone, desoxycorticosterone, prolactin preparations, saline extracts of the pituitary or an impure adreno-corticotrophic extract. Mammary growth was not caused by oestrogen in combination with gonadotrophic extract, thyrotrophic extract or saline extract of human placenta. No purified or partially purified pituitary hormone caused mammary growth when used alone, but injected saline extracts of the pituitary or implanted pituitaries sometimes stimulated growth. The importance of the adrenotrophic hormone has been shown by Nelson (104), who observed that when this hormone was administered to male and female rats mammary development occurred in the absence of the gonads and pituitary, but not in the

absence of the gonads and the adrenals. Adrenotrophic hormones contained in an anterior pituitary extract and administered with oestrone or follicle-stimulating hormones to hypophysectomized rats by Lyons, Simpson & Evans(105) caused marked lobule-alveolar growth.

Many of these latter experiments do not support the important 'mammogenic' theory of Turner. To account for this lack of agreement in much of the evidence some of the research workers involved(102) have suggested that although the mammary gland may be stimulated to develop by the action of oestrogens alone, the presence of pituitary secretions aids the development either by sensitizing the mammary tissue to the action of oestrogens or by improving the general condition of the animal with the result that mammary development proceeds more readily. Others(94) have suggested that some concomitant sequel of hypophysectomy rather than the loss of the pituitary itself may frequently be responsible for preventing mammary development in hypophysectomized animals and may thus give rise to erroneous conclusions. Turner and his colleagues, who are mainly responsible for the pituitary mammogenic theory, suggest that where the local application of oestrogens causes stimulation of the treated gland alone, sufficient Mammogen I may be circulating in the blood to cause duct growth in all the glands, but that the lack of duct growth in the untreated glands is due to the absence of some local action of the oestrogen such as the production of local hyperaemia or the maintenance of a large blood supply round the treated gland. In this connexion oestrogens evidently have a much greater power of providing the necessary local stimulus than ordinary skin irritants such as turpentine(93, 106). The views of Turner and his colleagues on the lobule-alveolar growth factor of the anterior pituitary have recently been explained in a bulletin(107) which is very well produced and excellently illustrated.

Bischoff & Ingraham(108) have studied the relative effects on mammary development of administering sheep pituitary extracts to rats subcutaneously, intraperitoneally and mixed with $ZnSO_4$ subcutaneously. The rate of absorption decreased in this order. Hypertrophy of the ovaries was proportionate to the delay of absorption. Delayed but not rapid absorption produced marked mammary a'veolar development. The relation of nutrition to mammary growth after oestradiol administration has been discussed by Samuels, Reineke & Petersen(109).

Since the discovery of the oestrogenic properties of stilboestrol and its derivatives, the stimulating action of these substances on mammary growth has been studied in some detail. They have been found to cause mammary development in female guinea-pigs by Nelson(110), in male guinea-pigs by Lewis & Turner(111) and in goats by Mixner(112), Lewis & Turner(113) and Folley, Watson & Bottomley(114). Stilboestrol therapy in farm animals is discussed in a subsequent section (p. 206).

Valuable observations on teat growth in immature female goats have been published by Folley & Bottomley(115). The ovary appears to display endocrine activity shortly after birth, for teat growth which is isometric immediately after birth became allometric when the animals were between 41 and 108 days old.

Lactogenic activity

(1) *The anterior pituitary.* Most of the numerous papers which have been published in the past few years on lactogenic activity describe experiments on small animals. Since the present review must be confined as far as possible to dairy cattle, reference will be made only to those experiments of which brief mention seems essential if the present position is to be understood.

At the outset it will be well to describe briefly a conflict of opinion which has arisen with regard to lactogenic activity. For many years the view has been held that the anterior pituitary secretes a specific lactogenic hormone which initiates and maintains lactation. This hormone has commonly been known as prolactin, but also as galactin or mammotropin. In the early work the extracts containing prolactin were also found to stimulate growth in the pigeon-crop gland, and on this observation methods of assaying prolactin activity were established, preparations rich in the pigeon-crop factor being assumed to be proportionately rich in the lactogenic factor. Folley & Young(116, 117, 118), whose work was discussed in detail in the previous biennial review of this series(119), suggested that such an assumption was untenable, for they found that they could prepare anterior pituitary extracts which had the power of stimulating growth in the pigeon-crop gland, but which would not enhance established lactation in the cow.

One crude extract they prepared was three times more potent in stimulating milk production than an extract possessing seven times as much power to stimulate pigeon-crop proliferation. Folley & Young suggested that the term 'prolactin' should be used to denote only pigeon-crop activity and that 'lactogen' should be reserved for substances which initiate or enhance already established lactation. Should it be desirable to do so, they also suggested that 'lactogen' could be further reserved for a hormone which stimulates the inititation of lactation and 'galactopoietic' for one which enhances already established lactation.

In spite of this work by Folley & Young, Bergman, Meites & Turner(120) still believe that the prolactin and lactogenic hormones are identical. They draw their conclusions from experiments in which they assayed a prolactin preparation at various stages of purification by the pigeon-crop method and also by a method based on the initiation of lactation in the pseudo-pregnant rabbit. Purification did not affect the ratio between the two types of unit. In citing this work in a lucid article on the subject, Folley & Young(121) have suggested that these findings may simply mean that by the methods of purification adopted two distinct hormones were concentrated in the same proportions. Folley has also pointed out that galactopoietic power probably depends on the co-operative action of more than one anterior pituitary hormone and that synergism between prolactin and glycotropin may be an important factor. In a later paper Bergman & Turner(122) have reported the results of tests on the effect of the prolactin fraction and 'thyrotropic and other hormone' fractions of the pituitary on lactogenesis in the pseudo-pregnant rabbit. They conclude that the primary function of prolactin, which in their opinion also possesses the ability to stimulate growth in the pigeon-crop gland, is to initiate and maintain established lactation. Extracts rich in thyrotropic and other hormones, but containing only traces of prolactin, were not able to initiate lactation, but had a supplementing effect on established lactation. Folley & Young(121), however, criticize this later work of Bergman & Turner on the following grounds. (1) Bergman & Turner dried their various fractions with alcohol and ether, which in the experience of Folley & Young, leads to the loss of much of the galactopoietic activity. (2) There was no evidence presented to show that Bergman & Turner's prolactin fraction was free from glycotropic factors. (3) The experiments were carried out on intact pseudo-pregnant rabbits whose own pituitaries may have provided complicating factors.

Whatever the ultimate outcome of this conflict of opinion, the matter is extremely important clinically, for, as Folley & Young(121) rightly point out, a lactating woman whose milk yield it is desirable to increase is probably much more akin to a cow in declining lactation than to a pigeon! It is therefore desirable that the true galactopoietic activity of extracts for clinical use should be assayed on mammals and not judged solely by their effect on the pigeon-crop gland. Until this difference is settled beyond dispute the term 'prolactin' will probably continue to be used, as in the past, to imply not only pigeon-crop stimulating properties but also lactogenic and galactopoietic properties. All three activities are implied when the term is used in many of the papers now to be reviewed. Before proceeding to these papers it will be well to emphasize here that the galactopoietic activity of the pituitary probably depends on several individual factors which include prolactin, glycotropin and other pituitary hormones.

Meites & Turner(123, 124) have carried out experiments on lactating rats, immature female guinea-pigs and male guinea-pigs to determine why lactation is not initiated during pregnancy and also to determine whether oestrogens suppress the secretion of the lactogenic hormone or whether they stimulate its secretion and are responsible for the great increase in the lactogen content of the pituitary which follows parturition. The following conclusions were arrived at. (1) Oestrogens do not inhibit lactation by suppressing prolactin secretion. If any inhibition of lactation does occur, it must be due to suppression of hormones other than prolactin which govern the level of milk precursors in the blood. (2) The rapid increase in pituitary prolactin content at parturition is due to the action of oestrogen no longer inhibited by progesterone. (3) Lactation does not begin during pregnancy because the ratio of progesterone to oestrogen is so great that the oestrogen cannot induce an increased secretion of prolactin. Valuable evidence that progesterone inhibits the secretion of prolactin by the pituitary whereas oestrogens do not, has also been advanced by Selye(125).

Lyons(126) has demonstrated the action of prolactin by a very ingenious method. He first induced lobule-alveolar growth in hysterectomized-ovariectomized female rabbits by oestrone

and progesterone injections and then injected prolactin into selected galactophores. Milk secretion was produced usually within 48 hr. but only in the sector of the mammary gland corresponding to the injected galactophores. No lactation occurred in uninjected animals or in control sectors injected with solvent.

Evans, Simpson & Lyons(127) claim that a purified prolactin preparation is the only factor which will activate corpora lutea which are not secreting progesterone owing to the removal of the hypophesis. They therefore suggest that the lactogenic hormone should be considered as belonging to the gonadotrophic complex since it possesses luteotrophic activity as well as mammotrophic and crop gland stimulating activities.

Much has been accomplished in the last few years on methods of extracting and separating some of the anterior pituitary hormones and in establishing their physical and chemical properties. A bulletin by Bergman & Turner(128) reviews the literature and describes the preparation and assay of 2·98 g. acetone-dried powder from 15 kg. of fresh bovine anterior lobe tissue. After the separation of other pituitary principles, the final preparation was relatively pure and contained 20,000 i.u. of prolactin per gram, adrenotrophin being the chief contaminant. Outstanding contributions describing fundamental work which should go far towards solving many of the chemical and physiological problems associated with prolactin and the pituitary hormones generally has been published by White, Bonsnes & Long(129) from Yale and by Lyons and his colleagues from the University of California. White *et al.*(129) have recently made a crystalline preparation of prolactin from bovine pituitary extracts by two methods. One method is similar to that already adopted by other workers for the crystallization of insulin from acetic-acid pyridine mixtures. The other method depends on precipitation from 80% acetone. The homogeneity and chemical similarity of crystalline and highly active amorphous preparations has been demonstrated by solubility, electrophoretic and ultracentrifugal experiments. The prolactin activity, estimated by the method of Lyons(130), showed that crude extracts contained 10–15 i.u. per mg. as compared with crystalline or highly purified samples prepared in three different laboratories which all contained approximately 30 i.u. per mg. The molecular weight, assuming the molecule to be spherical, was calculated to be 35,000. The iso-electric point was between 5·65 and 5·70. The analytical figures for C, H, N, S and ash are recorded for various samples. The average values approximated to 51·5, 6·9, 16·5, 2·0 and 0·6% respectively. The contents of tyrosine, tryptophane and cystine were found to be 5·5, 1·3 and 3·4%. The prolactin activity was destroyed by heating the crystalline protein for 15 min. at *p*H values greater than 9. After 30 min. it was destroyed at all *p*H values from 1 to 13. Hydrolysis with acid and with pepsin or trypsin rapidly destroyed the prolactin activity which therefore depends on the protein nature of the compound.

Lyons and his colleagues in California had already shown that free amino groups were essential if the hormone activity was to be retained(131, 132). They have since made a most thorough examination of the effect of iodine on the protein(133, 134) and have shown that in combining with prolactin, iodine reacts only with the tyrosine portion of the molecule, and that in doing so it inactivates the hormone. Tyrosine is therefore essential as well as free amino groups. Lyons and his colleagues also concluded that no free —SH groups are present in aqueous solutions of their prolactin preparation. Solubility studies(135) showed that prolactin prepared from sheep and bovine pituitaries were not identical although both behaved as pure substances. The fact that they are different was confirmed(136) by their tyrosine contents which were 5·7% for bovine prolactin and 4·5% for sheep prolactin. The tryptophane contents for both were first found to be 1·2 or 1·3%, but later(137) these values were corrected to 2·5% for both types. The authors point out that prolactin is therefore probably species specific. In later work, however(138), they found no difference between sheep and bovine prolactin in iso-electric point and electrophoresis data. By osmotic pressure measurements(137) the molecular weight of the crystalline product from both sources was found to be approximately 26,500, while from the cystine, arginine, tyrosine, tryptophane and sulphur contents it was estimated to be between 24,000 and 25,600. In a later paper of this series of publications on prolactin, Lyons and his colleagues(139) describe a new method for isolating the crystalline protein from sheep pituitaries. Its solubility, electrophoresis and diffusion properties showed it to be a single substance. Its specific rotation was $-40{\cdot}5°$ at 25° C.(140). Viscosity data showed that the molecule was not

spherical, and from the viscosity and density data the molecular weight was computed to be 22,000. The sulphur content of both bovine and sheep prolactin was found to be entirely accounted for by the 3·1% of cystine and 4·3% of methionine which they contain (141). Fraenkel-Conrat *et al.* (142, 143) have studied the effect of thiol compounds on the activity and reducing groups of prolactin. They found its cystine content to be 3·0%.

Much work continues to be published on the assay of prolactin. Riddle, Bates, Miller, Lahr, Smith, Dunham & Opdyke (144) have emphasized that the intradermal micro-test based on pigeon-crop gland experiments must be used with great care. In a series of tests with a variety of non-specific substances such as laboratory reagents and solvents, one injection of 0·5 ml. of many of the substances caused cell proliferation in the crop, sometimes with ulceration. Even beef muscle extract was shown to increase the crop-gland response (145). The observation has also been made (146) that the crop-gland response to a constant dose of prolactin given at regular intervals over a period of 2 years was highest in the winter and summer months and least in the periods from April to May and from October to November. When crop-sac weights were converted to equivalent units of prolactin by a dose response curve, the values found during winter and summer were three times as great as those obtained during the shorter spring and autumn periods. An assay by the crop-gland method should therefore be accompanied by an assay on standardized prolactin. The reason for this semi-annual cycle in crop responsiveness is not known, but it does not appear to be related to seasonal variations in body weight. Cary (147) reports a seasonal variation in the amount of prolactin present in the bovine pituitary. It was 400 or 600% greater in spring than in August.

Meites, Bergman & Turner (148) have compared assay by crop-gland response with assay by induction of lactation in rabbits, and have concluded that the factors determined by the two procedures are identical. They also used (149) international standard lactogen with the crop-gland procedure to compare subcutaneous injections with shallow intrapectoral and intradermal injections. The amount of lactogen required to give a response equal to 1 unit was 0·1 mg. subcutaneously, 0·125 mg. intrapectorally and 0·0006 mg. intradermally. Two-, three- and five-fold differences in the volume injected intradermally made no difference to the response obtained from a given amount of hormone. This finding conflicts with that of Bates & Riddle (150), cited in the previous review (119), who found that the volume of fluid injected greatly affected the response.

Riddle's method of assay has been discussed by Uyei, Itoh, Hayatsu & Uyeno (151) and other methods by Cary (152) and by Hall & Nicolet (153). A very promising method of assay has been described by Lyons (130), who uses virgin guinea-pigs of over 6 months old or 650 g. in weight. Within 3 days of oestrus, four daily subcutaneous injections are given and the response is judged by the occurrence of lactation within 24 hr. of the last injection. By this method the purified prolactin studied by Lyons was twice as potent as the international standard. By the pigeon test it was three times as potent.

The application of logistics to the response of the crop gland to prolactin and other biological assays has been discussed by Emmens (154).

An increase in the prolactin content of pituitaries of small animals has been shown to be caused by oestrone (155), by oestrogens and progesterone together (156), and by stilboestrol (157). With androsterone (158) no significant change in the prolactin content was observed.

(2) *The adrenals.* Since the adrenal glands are so essential for the maintenance of the salt balance and normal carbohydrate metabolism in the body, it is not surprising to find that adrenalectomy prevents lactation. There is, however, some evidence to show that the adrenals in some way affect lactation more specifically than simply by their influence on the salt and glucose levels in the blood. The importance of the adrenal cortical hormone for the initiation of lactation was shown some years ago by Nelson & Gaunt (159) and by Reece (160). Since then experiments carried out by Tobin (161) have shown that NaCl drinking solutions, crystalline progesterone, crude progestin, testosterone propionate and Antuitrin S did not enhance lactation in the doses which he used on adrenalectomized rats. Gaunt (162, 163) has confirmed the necessity of adrenal cortical secretions and found that desoxycorticosterone acetate, unlike adrenal cortical extract, was of no benefit in correcting the deficiency. This has also been shown by Nelson (164). In later experiments Gaunt, Eversole & Kendall (165) found that all substances

known to relieve adrenal insufficiency were somewhat helpful in maintaining lactation in adrenalectomized rats, but normal lactation was only obtained with whole extract of adrenal cortex or with 17-hydroxy-11-dehydro-corticosterone. Dehydrocorticosterone effected some improvement, but the results with desoxycorticosterone were very erratic. The authors suggest tentatively that normal lactation is dependent on those adrenal factors which primarily affect carbohydrate metabolism. Spoor, Hartman & Brownell(166) have described a method of preparing 'cortilactin' from whole adrenal glands by iso-electric precipitation at *p*H 5·8 in the presence of 86% alcohol. This substance, like prolactin from the pituitary, possesses power to stimulate crop-gland proliferation in the pigeon. The best preparation was about one-tenth as active as purified prolactin in this respect. Cortilactin was found to maintain normal lactation in adrenalectomized rats, but since prolactin itself would not do this, the two are not identical. Cortilactin was found not to contain the glycogenetic factor of the adrenals. The adrenals therefore appeared to have some specific lactation factor not directly associated with carbohydrate metabolism. These findings led Hurst, Meites & Turner(167) to assay various adrenal preparations for prolactin activity by a method which they claim to be 160 times as sensitive as that used by Spoor and his colleagues. They could find no evidence of prolactin activity in two commercial adrenal cortical extracts in whole untreated rabbit adrenals, and in iso-electric precipitates from beef, pig and rabbit adrenals. They suggest that the method of assay used by Spoor *et al.* may have been at fault in that the response was estimated by the increase in weight of the crop gland as distinct from actual proliferation of the crop-gland mucosa. Meites *et al.*(168, 169) have carried out experiments to find whether adrenalectomy affects the lactogenic secretory activity of the pituitary. In thirty-three rats adrenalectomy only reduced the lactogen content of the pituitary by about 26%. Adrenalectomy during the last week of pregnancy did not prevent the great rise which normally occurs in the lactogen content of the pituitary at parturition. The failure of the adrenalectomized rats to lactate was therefore not due to insufficient lactogenic hormone in the pituitary, a suggestion which is supported by the inability of prolactin to stimulate lactation in the absence of the adrenals. Turner & Meites(170) found that desoxycorticosterone given to male and female guinea-pigs caused an increase in pituitary weight but not in the amount of prolactin per pituitary gland. Contributions on the functions of adrenal hormones have also been made recently by Climenko & McChesney(171) and by Laszt(172). Folley(173) has found that doses of desoxycorticosterone as great as 5 and 10 mg. given daily with progesterone did not inhibit lactation in rats.

The thyroid gland

Following on the work of Leonard & Reece(174) in which thyroidectomy was found to cause enhanced mammary development in the rat, Smithcors & Leonard(175) carried out a series of experiments which led them to conclude that thyroidectomy in immature rats treated with oestrogens leads to an inhibition of mammary duct extension, the production of a small compact type of gland with thickened ducts and a marked development of lobule-alveolar tissue. Mixner & Turner(176), experimenting with mice, found that thyroxine treatment significantly increased the percentage of castrate mice which showed mammary lobule-alveolar growth when treated with oestrone and progesterone. They also found, contrary to the results of Leonard and his colleagues with rats(174, 175), that thyroidectomy under the same circumstances inhibited mammary growth. The authors point out that as mammary proliferation of this type is mainly protein in nature, the enhanced growth which they obtained with thyroxine treatment may be the result of superimposing the general protein growth effect of thyroxine upon the more specific mammary growth stimulating effect of oestrone and progesterone. The general increase in cell metabolism as a result of thyroxine may also in itself contribute towards increased proliferation in the presence of the other two hormones. Mammary duct proliferation was observed by Gardner(177) in thirteen out of fourteen intact male mice fed desiccated thyroid (1·5 g./kg.). This effect was prevented by castration.

Turning from mammary growth to lactation itself, Folley(178) has confirmed his earlier work which showed that thyroidectomy inhibits lactation in rats. He emphasizes that the inhibition is partially due to parathyroid deficiency, a fact which was confirmed by later experiments(179). The effect of thyroidectomy on lactation in the cow has been studied by Spielman, Petersen

& Fitch(180). Two cows were thyroidectomized during early lactation. Milk production practically ceased after 110 days and there was a total reduction of about 70% in milk-fat production compared with the previous lactations. In three cows thyroidectomized before conception, lactation ceased after 195 days and again total production was greatly decreased. No significant changes in the composition of the milk or blood were observed. Feeding fresh thyroid gland tissue to thyroidectomized cows restored their milk and fat outputs provided the interval between thyroidectomy and the beginning of the thyroid feeding was not too great. The action of thyroxine in enhancing milk and milk-fat secretion does not seem to be caused by an increased secretion of prolactin for, according to Reineke, Bergman & Turner(181), thyroidectomy in young male goats does not lead to an abnormal content of lactogenic hormone in the pituitary, nor does thyroxine when injected into lactating goats increase the excretion of lactogenic hormone in the urine(182) in spite of the fact that it increases the milk yield.

The stimulation of mammary development and lactation in farm animals by hormones

The two principal factors which have always tended to prohibit the use of hormone therapy for increasing milk production are the expense and rarity of the various preparations and the fact that they often require to be given by injection. The recent discovery by Dodds, Goldberg, Lawson & Robinson(183) that certain relatively cheap stilbene derivatives possess oestrogenic activity has caused the application of these substances, particularly diethylstilboestrol, to be studied in detail during the past few years. Thus, in recent experiments with goats, Lewis & Turner(184, 185) injected two female kids and two yearling female goats daily with 0·25–0·5 mg. stilboestrol. The milk yield reached 100–200 ml. per day from the kids and 500–600 ml. from the yearlings. At the same time the urine of the yearlings contained twice as much prolactin as that of dry yearlings and as much as that of goats which had kidded normally. It is well known that stilboestrol applied in sufficiently large doses does not promote lactation but actually inhibits it. A possible explanation for this has been advanced by Turner & Meites(186), who have found that large applications of stilboestrol cause enlargement of the adrenal glands and the secretion of cortin which increases the utilization of proteins and amino acids for energy purposes and causes an increased urinary excretion of N.P.N. In these circumstances availability of the precursors of milk protein may be greatly reduced. The theory also accords with the changes at parturition, when milk secretion is stimulated and adrenal cortex activity declines, unless the placenta is retained when depression of lactation and adrenal hyperplasia are observed. By this theory large doses of natural or synthetic oestrogens may be expected to inhibit lactation while smaller doses which stimulate the pituitary to secrete mammogenic and lactogenic hormones may be expected to result in mammary development and enhanced lactation.

Folley, Watson & Bottomley(187) have made a very extensive investigation of the effect of stilboestrol on milk yields in the goat and have concluded that the oestrogen stimulates the pituitary to secrete lactogenic and galactopoietic hormones. Several goats were used, but special reference may be made to twin animals which were treated identically except that the udder of one was rubbed with 1 g. of 1% stilboestrol ointment three times weekly while only basal ointment without any oestrogen was used for the other. Milk secretion began after about 8 weeks with the first animal and after about 28 weeks with the second. The yield of the first animal rose rapidly and remained at nearly 2 l. per day for 28 weeks. The yield of the second goat rose much more slowly and did not exceed about 1 l. In the early stages of artificial lactation the changes in milk composition were similar to those characteristic of the colostrol period. Thereafter the milk was of excellent composition. It appeared from these results, contrary to the finding of de Fremery(188), that for lactation in the goat, application of oestrogens is sufficient and that prolactin treatment is not required. Later work, however, suggested that the administration of prolactin may often be beneficial, even if it is not absolutely essential. Thus, in further experiments Folley & Young(189) found that for one goat a daily yield of 570 ml. induced by oestrogens was increased to 870 ml. by anterior pituitary injections, and that with another goat, in which a daily yield of 2 l. had resulted from oestrogen alone in the first year, the same yield was attained in the second year only when prolactin was also administered. The value of giving anterior pituitary injections as well as oestrogens is also discussed by Trait & Kirchoff(190).

The induction of lactation by stilboestrol dipropionate in a 3-year-old barren goat which ultimately gave a yield of over 2 qt. daily is reported briefly by Cockburn(191).

Stilboestrol therapy has also been applied to heifers and cows. Folley, Watson & Bottomley(192) found that with virgin heifers inunction with oestrogenic ointment only produced small quantities of a colostral secretion. Reece(193), however, reports that injections of diethylstilboestrol alone and in conjunction with testosterone propionate were successful in stimulating milk secretion in two nulliparous heifers, but it was found that a suckling calf aided in raising the milk yields. Peak yields of 27·5 and 33·7 lb. per day were recorded with average fat tests of 3·8 and 5·1%. Walker & Stanley(194, 195) have also described the stimulation of mammary development and lactation in heifers. In a 1-year-old spayed heifer and a 3-year-old sterile heifer injection of oestrogen caused maximum daily yields of 16 and 14 lb. Again it was confirmed that small doses of oestrogens stimulate mammary growth and lactation while larger doses inhibit both. Stanley & Owen(196) found that the injection of a total dose of 110 mg. stilboestrol injected into a lactating cow decreased milk production by 23% compared with a spayed cow similarly treated. They suggest that this effect might be used as a method for measuring the output of oestrogen by the cow's ovary. The production of 11½ lb. of milk daily by a 3-year-old barren heifer by injection of stilboestrol is briefly recorded by Kochan(197). A very brief mention is made in the *Journal of the Ministry of Agriculture*(198) of the recent work carried out in Britain by the Agricultural Research Council on the use of stilboestrol. The Agricultural Improvement Council has made arrangements for wider trials to be made throughout the country.

Articles on the stimulation of mammary development by stilboestrol treatment have been written by Folley(199, 200, 201) and Lewis(202).

In 1936 Folley(203) showed that the administration of oestrogens to lactating cows caused an enrichment of the milk which tended to be accompanied by a slight decrease in yield, the total output of milk solids by the gland being increased. The availability of diethylstilboestrol as a cheap but active oestrogen and the possible value of such an effect where cows persistently secrete milk which does not come up to the legal standards led Folley, Watson & Bottomley(204) to explore the possibilities of using diethylstilboestrol and its propionate to cause enrichment of milk without decreasing the yield. The results obtained varied with cows of different breed and with different methods of administration. Oral administration with one cow led to no marked effect on milk yield or composition, whereas with the same cow injections led to a slight rise in non-fatty solids. Inunction brought about a marked increase in milk solids with no change in milk yield in one Shorthorn cow. The same treatment caused a slight increase in solids and a slight fall in milk yield in a Guernsey cow, but no significant effects were observed with four pregnant British Friesians. On increasing the dose, two of these Friesians aborted. The most favourable results were obtained by subcutaneous implantation of diethylstilboestrol and subcutaneous injections of an aqueous suspension. In a Shorthorn cow, both these methods resulted in a substantial and prolonged increase in the secretion of milk solids with no decline in milk yield. In three Ayrshires, however, the increase in solids following injection of an aqueous suspension was accompanied by an appreciable decrease in yield. Breed may be an important factor, for on the whole the best results were obtained with Shorthorns. The danger of abortion, the possible difficulty of getting the cow into calf after treatment with oestrogens and the danger of oestrogens being secreted in the milk are discussed by the authors. Little is known about the secretion of diethylstilboestrol in the milk, but the problem is being further investigated. Diethylstilboestrol has been injected intramuscularly into four cows by Spielman, Ludwick & Petersen(205). Marked increases in the fat and sugar contents of the blood and milk occurred without any decrease in protein content or milk yield. 50–150 mg. per cow gave the best results. The effect began 3 days after beginning the injections and lasted for 48–72 hr. after the injections ceased.

From work cited in earlier sections of the present review it is obvious that lactation is stimulated by more than one hormone. This view gains confirmation from experiments by Sykes, Meuleman & Huffman(206) in which injections of an anterior pituitary preparation possessing marked liver-fat activity resulted in very substantial increases in milk-fat production by the mammary glands of three cows. A fourth cow did not respond. The increase in fat production,

almost 100% in one cow and almost 50% in the other two, was mainly due to an increase in the percentage of fat in the milk.

The statement has already been made that two of the principal factors prohibiting the use of hormones for increasing milk production are the expense and scarcity of the active principles. As far as thyroxine is concerned these two problems are gradually being solved as a result in the first place of the discovery by Ludwig & Mutzenbacher(207) that when casein is treated with iodine under certain conditions, the resulting product possesses thyroid activity, a fact which was later confirmed by Harington & Rivers(208). Turner and his colleagues have devoted much work to the preparation and nature of this activated protein which they term thyrolactin. They have introduced an improved and economic method for its preparation and have shown(209) that the product they obtain restores normal growth in young thyroidectomized goats, that it produces 1% of the response of an equivalent weight of thyroxine in increasing the metabolic rate of guinea-pigs, and 2·7% of the thyroxine response when injected into tadpoles. They have also shown that it causes substantial increases in the yield of milk and butterfat when fed to cows(210). The literature on this subject and all but the latest experiments with iodinated protein have been discussed by Reineke & Turner(211) in another excellent bulletin from Missouri to which detailed reference should be made. When 5–10 g. were fed daily to goats increases in milk production averaging 10·5% and reaching a maximum of 41% were obtained. At the same time an increase of 8·2 beats per minute was observed in the heart rate(212). 50–100 g. fed daily to cows caused average increase in milk yield of 8·6%, individual increases varying from 6 to 23%. Some of these facts are also dealt with in an article by Reineke & Turner(213) on synthetic thyroprotein in veterinary practice. In this country Blaxter(214) has carried out feeding trials with two iodinated protein preparations, the trials involving 208 cows. One preparation 'Ardein', fed at the rate of 50 g. daily, increased milk production by 35% and fat production by 54%. Iodinated casein at the rate of 15 and 30 g. daily gave increases in milk yield of 16 and 33% respectively. The responses were therefore similar to those which can be obtained with thyroxine itself. Clinical abnormalities shown by the cows even on the high dosages were not severe and weight losses were soon regained on cessation of treatment. Van Landingham, Henderson & Weakley(215) have also reported marked increases in milk and fat production on feeding iodinated protein to two groups of Holstein cows, but they found that the ascorbic acid content of the milk decreased by about 33%. Full details for the preparation of the iodinated protein used by the American workers will be found in papers by Reineke and his colleagues(216, 217) who have now succeeded(218, 219) in isolating *l*-thyroxine from iodinated casein after direct hydrolysis with a mixture of sulphuric acid and butyl alcohol. The yield of the laevorotatory crystalline hormone by this new procedure was 0·1%.

Thyroxine ingested by the ruminant tends to be destroyed in the rumen. To overcome this difficulty a patent has now been registered(220) for the preparation of pellets consisting of desiccated thyroid coated with some substance or substances which are believed to be insoluble in aqueous solutions at the *p*H of the rumen but which will dissolve at the higher *p*H of the small intestine. The pellets weigh 0·1–5 g. and are reported to have given good results.

Administration to cows of dinitrophenol(221) in doses which were too small to affect the heart or respiration rates, did not affect the milk yield but increased the yield and percentage of butterfat. When doses were given which were large enough to increase the respiration rate by 41% and the pulse rate by 55% marked decreases in the yield of both milk and butterfat resulted.

Factors involved in the ejection of milk

In some excellent experiments on the ejection of milk Ely & Petersen(222) have extended their earlier work which was cited in the previous review(119). In three non-lactating Jersey cows the left half of the udder was denervated and the ejection of milk by this half of the gland was compared with that by the intact half when the cows were subject to fright, or to injections of adrenalin, pitocin (the oxytocic principle of the posterior pituitary) and pitressin (the presser principle of the posterior pituitary). The results showed that milk ejection does not appear to be under the *direct* control of the central nervous system, for denervation had no effect either in the appearance of the gland or in the rate of milk ejection. Both fright and adrenalin injec-

tions resulted in cessation of milk ejections, the length of time for which the milk was held up depending on the amount of adrenalin injected. Intravenous injections of pitocin and pitressin even following fright or adrenalin treatment resulted in more complete drainage of the gland than could be obtained in normal milking. Pitocin appeared to be more potent in inducing ejection than pitressin, and it is suggested that the activity of the latter may have been due to incomplete separation of the pitocin. The extra milk obtained by pitocin contained 7·6–24·0% of fat but was otherwise practically normal. It is important to observe that the extra drainage of milk and fat brought about by the posterior pituitary factors resulted in lower yields of milk and fat at the next milking. Ely & Petersen concluded that many sources of afferent stimuli such as handling the teats, the rattling of milk buckets, the washing of udders and other factors associated with milking may stimulate the pituitary through the central nervous system to secrete oxytocin into the blood. The oxytocin then increases the intra-glandular pressure and squeezes the milk from the alveoli and smaller ductules. The process is thus a conditioned reflex. Afferent stimuli of a different nature, such as fright, can cause the secretion of adrenalin which has the opposite effect and prevents the ejection of the milk. Miller & Petersen(223, 224) have shown that delay between exciting the stimulation of oxytocin and the completion of the milking process results in a decreased yield which is probably due to the gradual dissipation of the oxytocin from the blood. The importance of the oxytocin content of the blood has been demonstrated by Petersen & Ludwick(225) who have drawn blood from cows which had been stimulated to eject their milk and used it to perfuse excised mammary glands from which milk had been drained as completely as possible. A further release of milk resulted from this treatment, whereas perfusion with blood from cows that had not been previously stimulated had no such effect. The active agent was destroyed when the blood was allowed to stand for half an hour. Petersen(226) has extended this work to determine the relative effect of various substances. Perfusion with oxytocin or acetylcholine caused complete ejection. With acetylmethylcholine, vasopressin, epinephrine or histamine ejection was only partial. Atropine and carbamylcholine had no effect. The result of continuous injection of pitocin upon milk and milk-fat production has been studied by Knodt & Petersen(227). Both milk yield and percentage of butterfat were increased during the injection period, but were markedly reduced when injections ceased. Shaw(228) has found that with the exception of the first day of injection and the day after the last injection, the injection of oxytocin at regular milking intervals did not significantly affect the amount of milk and fat secreted. Milking with the aid of oxytocin at 2-hourly intervals demonstrated that the rate of milk secretion is extremely constant and is not influenced by the frequent removal of the milk from the gland. The total milk production was unaffected by the frequent removal of the milk, but the milk-fat secretion decreased by 11% in 24 hr. A method of assaying the posterior pituitary factors which are involved in the ejection of milk has been suggested by Turner & Cooper(229). It is based on the minimal amount of substance which produces a minimal contraction of the mammary gland in 40 sec. when injected into the ear vein of a lactating rabbit. The effect of pitocin on the lipolytic activity of mammary gland tissue has been discussed by Kelly(230).

Ely & Petersen(222), in the work cited above, suggest that suckling may be one of the afferent stimuli which cause the posterior pituitary to secrete oxytocin which in turn lets down the milk. The effect of suckling on the secretory process as distinct from ejection is discussed in a later section (p. 217).

A review on the subject of hormones and lactation was published by Petersen in 1942(231).

(2) *Biochemical aspects*

During the past four years since the previous review(119) was prepared, further experiments have been carried out which involve the principle of obtaining representative samples of blood entering and leaving the lactating mammary gland with the object of determining the nature of the milk precursors and the amounts in which they are used by the gland. Two groups of research workers have been engaged in these studies, Shaw, Petersen and their colleagues at Minnesota and Turner and his colleagues at Missouri. In work of this type it is essential that the experimental animal should not be excited or unduly disturbed during the blood-sampling

period. With the object of avoiding disturbance Reineke, Williamson & Turner(232) have studied the effect of nembutal anaesthesia on the secretion and composition of goat's milk. They found that milk of normal composition is secreted at a normal rate and that the uptake of glucose from the blood entering the mammary gland continues during the nembutal treatment. Turner and his colleagues claim therefore that results obtained by this means are more reliable than results from non-anaesthetized animals since artefacts resulting from disturbance and excitement are avoided. Petersen, Shaw & Visscher(233), with a similar object in view, have developed a technique in which excised lactating mammary glands are perfused with blood and the uptake of milk precursors estimated. They give a detailed description of their method with an illustration of the apparatus and full directions for excising and perfusing the gland. Contact of the blood with metal must be avoided, the blood must be filtered from small clots and the blood pressure must be kept constant. Shaw & Petersen(234) have also studied arterial-venous sampling in the intact animal and have discussed their method and the effect of disturbance upon it.

By these various methods valuable information on the precursors of milk has been obtained in recent years. Thus, experiments by Reineke, Williamson & Turner(235) have shown that glycoproteins of the blood are taken up by the lactating mammary gland. On the average 2·15 mg. glycoprotein was removed per 100 ml. plasma by the mammary gland of a lactating goat compared with values of 0·38 mg. for a non-lactating non-pregnant goat and 0·58 mg. for a non-lactating pregnant goat. Since the glycoproteins of the blood occur only in the globulin fraction, this finding is suggested as further evidence that globulin is one of the precursors of milk solids. It may be recalled here that Hammond(237) suggested many years ago that the precursors of both sugars and proteins in milk might be glycoproteins of the blood and that Smith, Howat & Ray(236) observed the presence of an unidentified constituent which was thought to be a glycoprotein in certain samples of abnormal milk. From further experiments on the amino-acid uptake of the mammary gland Reineke *et al.*(235) have suggested that the globulin fraction of the blood is the main precursor of milk protein and that free amino acids may not be essential for the formation of milk.

Earlier work(238, 239) had suggested that lactic acid was removed by the lactating gland as one of the precursors of milk. Powell & Shaw(240) have now studied this matter further and have come to the conclusion that the apparent decrease in the lactic acid content of the blood as it passes through the mammary gland occurs only when excitement has caused a sudden increase in blood lactic acid which is naturally followed by a rapid diffusion of the lactic acid into the glandular tissue. It does not occur when the animals have been anaesthetized for more than 30 min. Lactic acid is therefore almost certainly not one of the precursors of milk.

Most of the recent experiments involving arterial-venous sampling have been devoted to a study of the precursors of the fatty constituents of milk. Voris, Ellis & Maynard(241) have devised a method of estimating from 0·1 to 2·5 mg. glycerol. They have used the method to determine the neutral fat content of blood, and have confirmed that neutral fat is taken up by the lactating mammary gland. Shaw & Petersen(242, 243) have found that in the absence of disturbing factors the average difference in fat content between arterial and venous blood was 9 mg. per 100 ml. which appeared to be sufficient to account for all the milk fat. They showed that little fat was taken up immediately after milking, but that the rate of uptake increased up to 4 hr. and then remained constant. This was true also for calcium, but not for glucose and amino acids. Oxytocin injections prevented the passage of fat even into the glands distended with milk. The utilization of ketone bodies by the lactating mammary gland and the formation of the lower fatty acids in milk have also been studied by arterial-venous sampling technique. In sixteen determinations Shaw & Knodt(244) found that an average of 2·49 mg. β-hydroxybutyric acid per 100 ml. blood were removed by the lactating gland as compared with an average of 0·025 mg. in six determinations for non-lactating glands. From calculations involving the ratio of blood flow to the volume of milk secreted, they showed that this uptake of β-hydroxybutyric acid would be sufficient to account for the formation of all the lower fatty acids in milk up to C_{14}. Such a process would also agree with the fact that the respiratory quotient (R.Q.) of the gland is usually found to be greater than unity. Since, however, this acid is so readily oxidized in tissues, its use for synthesis of fatty acids by the mammary gland is suggested with very great reserve. The alternative is that β-hydroxybutyric acid is oxidized

in the mammary gland to supply energy. If so, 42% of the total oxygen consumption of the gland would have to be used for the oxidation of this compound, and to explain the high R.Q. in these circumstances there would have to be considerable synthesis of fat from carbohydrate. In extending this work Shaw & Knodt(245) observed that during inanition and when cod-liver oil was fed, the mammary R.Q. was less than unity, the Reichert-Meissl value of the milk fat was decreased and the amount of β-hydroxybutyric acid absorbed was reduced. In severe ketosis they found that the blood sugar was decreased to less than 50% of the normal value and that the mammary R.Q. was greater than unity. From these findings they concluded that the lower fatty acids are formed from β-hydroxybutyric acid and that carbohydrate is probably not used for fat synthesis. In a later paper, however, Shaw(246) emphasizes that this suggested formation of lower fatty acids from β-hydroxybutyric acid must be treated purely as tentative, and after further work(247) in which he found that the amount of β-hydroxybutyric acid taken up by the mammary gland of cows in ketosis was twice as much per 100 ml. of blood as with normal cows, although the amount of lower fatty acids in the milk was decreased, he concluded that the lower fatty acids do not arise from β-hydroxybutyric acid, but that they probably result from the oxidation of higher fatty acids as previously suggested by Hilditch and his colleagues(248, 249). He also concludes from his data that in the normal lactating gland some 37% of the oxygen consumed is required for the combustion of β-hydroxybutyric acid and 63% for the oxidation of fat, and that it is from these two sources that the gland obtains its energy. Acetoacetic acid and acetone do not appear to be utilized by the lactating mammary gland.

The fact that the R.Q. is usually found to be greater than unity while β-hydroxybutyric acid and so much fat are being oxidized has not yet been explained. There are certainly great difficulties involved in the estimation and interpretation of R.Q. Some of the difficulties can be seen from the wide variations which were obtained by Petersen & Shaw(250) for the R.Q. of both lactating and dry intact and perfused glands. For the lactating intact gland, for example, the oxygen uptake varied from 2·7 to 7·0 vol. %, while the values for CO_2 elimination varied from 1·5 to 9·3. In a more extensive series of experiments by Shaw, Powell & Knodt(251) many of the previous results were confirmed and it was estimated by Ca and P determinations that 494 volumes of blood traverse the gland for each volume of milk. On this basis the blood fat removed by the gland would account for 92% of the milk fat secreted, while the blood sugar removed would account for 105·5% of the lactose. This figure of 92% seems to be very low in view of the earlier conclusion(247) that a portion of the fat taken up is used for energy purposes.

Before this more recent work of Shaw *et al.* was published, Reineke, Stonecipher & Turner(252) carried out experiments which led them to maintain their belief that part of the fat in milk, more particularly the lower fatty acids may be synthesized from carbohydrates. They determined the R.Q. for the mammary gland of lactating goats anaesthetized by nembutal and obtained very constant values of 1·09, a value which became 1·18 on correcting for urea formation in the gland. They suggest that the big variations in oxygen uptake and R.Q. previously obtained by others in unanaesthetized animals were due to variations in the rate of ventilation of the lungs. When the animals were forced to fast, the R.Q. declined and passed below unity by the third day. When this fact is considered together with the decreased output of lower fatty acids observed during inanition by Smith & Dastur(253) it would appear possible that the lower fatty acids may be formed by synthesis from carbohydrate. There is no doubt, however, that since the difficulty of interpreting R.Q. measurements for a lactating gland, in which only a few of the many reactions are known, is very great indeed, the balance of the evidence at present available favours the hypothesis that the lower fatty acids arise from the oxidation of higher fatty acids. Shaw wisely suggests that judgement on the interpretation of R.Q. should be withheld until more is known of the complex reactions which proceed within the gland.

Passing now to other experimental methods which are used for investigating milk-fat problems, the feeding of sodium butyrate to a goat and a cow and of large amounts of silage as a source of butyric acid(254) led to no increase in the content of lower fatty acids in the milk fat. This suggests that the lower fatty acids of milk do not arise from lower fatty acids formed in the rumen, in spite of the fact that cows' and goats' milk fat contains so high a proportion of

lower acids as compared, for example, with human milk(255) in which the proportion of lower acids is extremely small. The work at Cornell carried out during the last 12 years by Maynard and his associates on the influence of dietary fat on milk secretion has recently been reviewed in a bulletin(256) in which more recent experiments are described. In the later work concentrate mixtures containing 3 and 7% fat were fed to two groups each containing eight cows and also to a group of nine cows. The double reversal procedure was adopted. The higher fat mixtures appeared to lead to an increase in milk yield of 4·4% in the first experiment and 1·1% in the second. The increases in yield of fat were 2·0 and 3·4%, and in fat-corrected milk they were 4·1 and 2·4%. Experiments by Loosli & Lucas(257) suggest that this type of increase is definitely due to the fat and not to additional vitamins which may be included in the high fat diets. Maynard, McCay, Loosli, Babcock & Lucas(258) intend to carry out metabolism studies to determine whether the beneficial results of the higher fat diets are explained by the dietary fat improving the digestibility of the other dietary constituents.

The effect of including several different fats in the diet of cows on the milk and fat production have been studied by Allen & Fitch(259). In 50-day periods, daily rations containing 1·5 lb. fat were compared with similar rations in which the fat was replaced by 3·4 lb. of a starch-sucrose mixture. No detailed results are given, but it is stated that the fat content of the milk and the yield of fat were markedly increased when butter fat, lard or tallow was fed: that they were increased with linseed, cottonseed and peanut oils, but that they were markedly decreased with corn and soya-bean oils. The depressing action of the last two oils is being investigated. In one of two feeding trials carried out by Munroe & Krauss(260) a high fat diet caused no significant difference in milk-fat yield, but in the other trial the high fat diet appeared to result in an increased secretion of fat. Cary(261) has carried out experiments to find how much cod-liver oil can be fed, without lowering the fat content of the milk. The results of feeding 50 and 100 ml. daily to each cow varied with different animals, but 30 ml. appeared to have no ill-effect with any of the cows. Prizker(262) has described investigations with ten cows to determine the proportion of fat in the different fractions of milk and how this is influenced by the milk yield, the age of cow, the level of nutrition, the month of calving and the time of milking. Successive litres of milk obtained at a milking were measured off and analysed separately. The higher the milk yield the greater was the difference between the fat content of the first and last fractions. Cows in their sixth to eighth lactations gave a higher proportion of fat than younger cows. A higher plane of nutrition and calving early in the year also resulted in a higher proportion of fat.

One method of investigating problems of milk-fat secretion has been to label the fats in some way. A recent paper by Huffman & Duncan(263) describes the effect of feeding fat-soluble dyes upon the colour of the milk fat. Five dyes were tested by feeding 15 g. of each dissolved in soya-bean oil. 12 hr. after feeding Sudan III and IV the milk fat was coloured pink. The colour reached a maximum 24 hr. after feeding and remained coloured for 144 hr. The colours produced by Brilliant Green and Perfect Purple did not appear in the milk fat so quickly nor did they persist so long.

A lecture by Sharp(264) on the fat globule should prove most informative. It begins in very popular style, but passes on in more serious vein to some of the important problems and peculiar properties associated with the fat globule.

Hilditch and his colleagues have continued their very extensive series of researches on the fatty constituents of milk. They have now analysed(265) the component fatty acids of milk fat produced by two groups of cows fed on fresh green pasture and by one group of cows fed solely on grass silage and hay during the winter season. The differences in composition of the three fats are discussed in detail. The fat produced on the silage diet contained more palmitic and hexadecenoic acid than the fat produced on pasture. It also contained less stearic acid (7·6% mol.) and oleic acid (22·4% mol.) than the fat obtained from the pasture-fed cows (10·1 and 28·3% mol. respectively). In these respects the 'silage' fat resembled the fat from cows fed on a winter diet of hay and roots (previously analysed by Hilditch and his colleagues(266, 267)), more than it resembled the fat from the cows fed on pasture. Since Hilditch & Jasperson believe that the proportions of lipins, carbohydrates and protein in the pasture diet of the summer must have been very similar to that in the silage diet of the winter, they conclude that

the change in the composition of the fat during the winter must be ascribed to some seasonal change such as temperature or difference in freedom of movement and exercise rather than to the change from pasture to silage. Further valuable evidence on this important point might be obtained by comparing the milk fat secreted by a group of cows fed silage with another group of cows fed either fresh grass or dried grass at the same time of the year and under the same conditions of management. In this series of experiments by Hilditch & Jasperson the polyethenoid C_{18} acids occurring in milk fat were studied. No evidence for the presence of the 'α'-linoleic (seed fat) acid could be found. In further work Hilditch & Jasperson(268) have studied the effect of dietary fat of varying unsaturation on the component acids of milk fat. Groups of three cows were fed on a basal ration consisting of hay, swedes, oats and groundnut meal and also on this ration supplemented with 8 oz. per day of the following fats, groundnut oil (i.v. 88), hydrogenated groundnut oil (two samples one with i.v. 45 and the other with i.v. 17) and palm kernel oil (i.v. 17). The detailed discussion of the results in the original paper and their bearing on milk-fat secretion should be consulted. Briefly it was found that the groundnut oil and hydrogenated groundnut oil (i.v. 45), which both contained more oleic acid than the control fat, led to increased amounts of oleo-glycerides and some decrease in butyric-decanoic glycerides in the milk fats. The more saturated oil increased the content of stearo-glycerides. Palm-kernel oil enhanced the proportions of lauro- and myristo-glycerides and slightly diminished both the palmito- and oleo-glycerides. The hydrogenated groundnut oil (i.v. 17) gave a milk fat almost identical with the control, an observation which is consistent with the fact that a large portion of this fat was made up of completely saturated tristearin or acyldistearin, since these constituents would have high melting-points and would thus be relatively unassimilable. There was no evidence for any desaturation of stearo-glycerides during transformation of hydrogenated groundnut oil to milk-fat glycerides.

Hilditch & Meara(255) have analysed the component fatty acids of human milk fat. They obtained only 0·45 and 1·73 g. of steam volatile acids from initial weights of 68·2 and 130·1 g. and in the volatile fraction they obtained no evidence for the presence of butyric, hexanoic or octanoic acids. The chief components were oleic (30–37%), palmitic (22–24%), stearic (8–9%), myristic (8–9%), lauric (5–7%), decanoic (2–3%), diethenoid C_{18} acids (about 7%) and unsaturated C_{20-22} acids (3–4%). Small quantities of hexa- and tetradecenoic acids were also present. The chief difference between cow and human milk fats is the absence in the human fat of acids lower than C_{10} and in the greater content of diethenoid C_{18} and higher unsaturated acids.

The fatty acids from two specimens of phosphatides isolated from Swiss and English butter fat clarification residues have been analysed by Hilditch & Maddison(269). The component acids as percentages of the total phosphatides were myristic 3·2, 5·5, palmitic 21·0, 13·4, stearic 7·3, 9·0, arachidic 12·3, 20·9, '$C_{26}H_{52}O_2$' 5·2, 10·0, hexadecenoic 4·3, 4·9, oleic 32·5, 23·5, octadecadienoic 6·4 and none, C_{20-22} unsaturated acids 7·8, 12·8. The two samples differed from each other in some details, but they were both very different from the glyceride fatty acids of milk fat. Except for their relatively low content of octadecadienoic and higher saturated acids they resembled in general the component fatty acids of bovine liver phosphatide as previously analysed by Hilditch & Shorland(270).

Milk energy yield and the correlation between fat percentage and milk yield have been ably discussed from a statistical point of view by Gaines(271, 272).

The use of radio-active elements for studying milk secretion has been adopted by a few workers in recent years. The secretion of radio-active iron has been investigated by Erf(273) and of radio-active strontium by Erf & Pecher(274). Strontium was used because its metabolism was believed to be similar to that of calcium whose only suitable radio-active isotope is not readily obtained in sufficient amounts. With two Holstein cows, 8 and 11% of the strontium was secreted in the milk during the 4 days following its injection. By far the greatest amounts in the milk occurred during the first few hours after administration, which is taken by the authors to suggest that much of the milk calcium is derived directly from dietary calcium. Further experiments with strontium on mice are described by Pecher & Pecher(275) and with selenium on rats by McConnell(276). The radio-active selenium appears to pass into the protein fraction of the milk where it is distributed mainly between the casein and the albumin.

Further biochemical investigations in recent years include a study of the *p*H of the bovine mammary gland by Kelly(277) who found the *p*H of the connective tissue to be 7 and that of the secretory tissue to vary from 5 to 7. Petersen & Brereton(278) have studied the effect of inhaling a variety of substances on the flavour of milk. The flavours of turpentine, *p*-dichlorobenzene, camphor and vanillin were all transmitted to the milk. Benzaldehyde, onions, garlic, corn silage and decomposing manure caused a change in flavour which was not characteristic of the administered substance.

The enzymatic hydrolysis of diacetin by bovine mammary tissue has been investigated by Kelly(279) and a comparison between the lipase content of goats' and cows' milk has been made by Lythgoe & Rosenthal(280).

Van Landingham, Henderson & Bowling(281) have continued their series of experiments on the chemical composition of the blood of dairy cattle. This recent work is concerned with the inorganic phosphorus of the blood and factors affecting it. In heifers the blood inorganic phosphorus increased from 6·58 mg. per 100 ml. blood at 1 month old to 7·15 mg. per 100 ml. at 6–8 months old. It then decreased gradually to 5·68 at 28 months. During the first gestation, values of 5·65 mg. P per 100 ml. blood at conception decreased gradually to 4·81 mg. at parturition. After the first lactation there was a tendency for the values to be lower during winter and early spring than in the summer and early autumn. The effect of parturition and early lactation on the carotene and vitamin A content of bovine blood plasma has been studied by Sutton & Soldner(282). A marked fall in the normal concentrations was observed a week before calving. The values reached a minimum 3 days after calving and then returned to normal during the following 4 weeks.

(3) *Anatomical aspects*

The milk yields and milking rates of the individual quarters of sixty-one Holstein and thirty-three Jersey cows have been recorded by Matthews, Swett & Graves(283). The highest yield was given by the right hind-quarter in 47·4% and by the left hind-quarter in 40·4% of the ninety-four udders. In 45·7% of the udders the lowest yielder was the left fore-quarter and in 29·8% the right fore-quarter. Variations in total milk yields caused by lengthening the periods between milkings, and variations in total milk yield occurring during one lactation and also from one lactation to another had no significant effect on the average relative milk yields from separate quarters, but milking rates (lb. per min.) varied not only during a single milking, but also from one milking to another if there were differences in total milk yields. Hind-quarters showed a higher maximum milking rate than fore-quarters.

The arrangements of the tissues by which the cow's udder is suspended has been described by Swett, Underwood, Matthews & Graves(284) in an article illustrated with eleven excellent photographs and one diagram. The secreting tissue of the udder is supported by seven distinct structures, the skin, the fine alveolar tissue, the coarse alveolar tissue, two lateral suspensory ligaments, two deep lateral sheets, the subpelvic tendon and a double sheet of yellow elastic tissue running medially. The authors hope that their description of this detailed dissection should make possible a better understanding of the condition that exists and the changes that take place when an udder has become 'broken down' or pendulous. From a study of twenty-five udders, Emmerson(285) concluded that the lateral suspensory ligaments form the chief mechanical support of the udder and that the external pudic vein is the most important channel by which blood leaves the udder. Emmerson also observed that supernumerary teats occurred in 44% of the 370 cows which he examined. The structure of the mammary gland of a champion cow which had produced 51,000 kg. milk in six lactations is described by Arzumanjan(286), who states that the ratio of glandular to connective tissue in this cow was 5·8:1 as compared with the ratio of 1:1 normally found in the average cow of the same breed.

The teat and gland cisterns have been described in detail by Veinzke(287) and Foust(288), the teat sphincter by Espe & Cannon(289), the structure of the milk ducts by Ziegler(290) and the capacity of the udder by Begouchev(291). Nogues & Gazzotti(292) have used the injection of colloidal thorium dioxide to examine the milk ducts by radiography. By suitable staining methods Swanson & Turner(293) have shown the presence of smooth muscle fibres beneath the secretory epithelium of the alveoli and in the interlobular spaces. It is believed that they aid

in the expulsion of milk from the lumen. The walls of the duct system also contained similar smooth muscle cells, but these do not appear to form muscular sphincters at the points where the ducts branch.

A study of the circulatory system of the udder has been described by Becker & Arnold(294) in an illustrated bulletin. They confirm previous findings that in most cows only two pairs of veins drain the blood from the udder. The bulletin includes diagrams showing the valvular distribution. In experiments by Espe & Cannon(295) an example of the re-establishment of the arterial blood supply to the udder has been shown. An inch of the right external artery was removed from a month old heifer. When the heifer came into milk no difference in milk yield or in appearance was observed between the two halves of the udder. It was found that the arterial supply to the right half was coming exclusively from the caudal branch of the left mammary artery. Genta(296) has studied the relationship between total milk production in lactation, intensity of production (i.e. maximum daily yield), and the size of the milk wells. 198 Normandy and 253 Shorthorn cows were used. For correlations between total production and intensity of yield, $r=0{\cdot}5697$ (Normandy) and 0·4816 (Shorthorn). For forty cows whose milk wells were measured correlations were obtained between intensity and production ($r=0{\cdot}6582$), between production and milk well surface ($r=0{\cdot}4148$) and between intensity of production and milk well area ($r=0{\cdot}4368$). Graves(297) has discussed data which he has collected over a period of 10 years and which have enabled him to correlate the development of subcutaneous veins on the surface of the udder and the size of the milk wells in the first lactation with the milk production in that lactation. What were regarded as good quality udders were not significantly correlated with development of milk veins or the size of the milk wells, and the size of the milk wells was not correlated with the prominence of the abdominal or udder surface veining.

The nerve supply to the bovine mammary gland has been described with the aid of excellent diagrams by St Clair(298), who has investigated the mechanism of milk ejection by direct stimulation and by the use of adrenalin and pituitary injections. It would appear here as in work already reviewed (p. 208) that in milk ejection sympathetic control is overshadowed by hormonal influence. The effect of section of the pituitary stalk on lactation in the rat has been described by Desclin(299) who finds that lactation is prevented when the stalk is severed.

The development of the bovine hypophysis has been described by House(300) and some of its morphological and functional relationships by Gilmore, Petersen & Rasmussen(301).

For recent data on the live weight and body measurements of cows and how these correlate with milk yield, reference should be made to the well-known work of Gaines and his colleagues at Illinois(302, 303, 304, 305, 306) and of Brody(307, 308) and also to data published by Axelsson(309), Mixner(310), Engeler(311), Kleiber & Mead(312), Krizenecky(313) and Myers(314, 315).

Graves(316) has studied the relationship between mammary gland development in the calf and milk-producing capacity in 130 Holstein and 130 Jersey cows. The calves were graded from an early age for mammary gland development and at the time of publication had completed their first lactation. The age at which the grade is most indicative of future milk-producing capacity appeared to be about 3 or 4 months. The relationship was not so good with the Jerseys as with the Holsteins. Data on the anatomy of the buffalo(317) and of the early development of the goat(318) have been published recently.

For semi-popular accounts of the cow's udder and its development reference should be made to articles by Petersen(319, 320, 321).

(4) *Miscellaneous factors affecting milk secretion*

One of the most topical studies made during the present war years has been the effect of air-raids on the composition of milk. The results have been reported by Rowland(322) and Arnaud(323). The incidents investigated occurred over a wide area of southern England and included a number in which high-explosive bombs had dropped in the midst of herds or sufficiently near them to kill or injure some of the animals. Some animals showed no signs of disturbance even when the explosions occurred within 100 yards. Others showed marked nervousness. A temporary reduction in milk yield frequently occurred, and sometimes milk

secretion practically ceased for several days. The composition of the milk, however, did not appear to be affected. No milk sample was encountered with less than 3·0% fat and the fat content did not alter significantly on successive days after the raid. The solids-not-fat content of the samples was also unaffected. During August and September 1940, the average figures of 3·64% for fat and 8·69% for solids-not-fat in the Ashford and Ramsgate area where many herds were bombed compared favourably with those of 3·68 and 8·75% for other parts of Kent.

The effect of altitude on milk yield has been studied recently by Engeler(324), who has drawn his conclusions from 22,800 annual records. Within a range of 500–1900 m. there was a decrease in yield of 65 kg. for every 100 m. increase in altitude. At the highest altitude the yield was 22% less than at the lowest. The decrease is ascribed to a number of factors such as poor vegetation, difficulties in management, greater mechanical and physiological strain on the animals and fluctuations in climate.

In the previous review(119) mention was made of experiments(325) which had been begun with the object of showing the folly of turning cows out of the byre during the day in extremely cold weather, but which led to the conclusion that provided the animals are sheltered from wind and snow and have dry bedding and adequate rations, their condition and milk production will not be adversely affected by exposure to extremely cold temperatures. Further evidence confirming this finding in two other places is published in a bulletin from Wyoming(326) and by Burger & Joubert(327) in South Africa. It was found from 4 years' results in Wyoming that during 30-day alternate periods in the winter, cows in open sheds gained weight and produced an average of 0·95 lb. butterfat, while during the periods in the byre, they tended to lose weight and had a daily butterfat production of 0·89 lb. This value was increased to 1·0 lb. daily for cows kept in open sheds throughout the year. Over a period of 3 years Burger & Joubert have studied the effect of keeping cows out in a sheltered field at night even when the temperature sometimes fell to as low as 14°F. No significant differences in the yield and composition of the milk or in the body weights and food consumption of the cows were observed for cows under open-air conditions at night compared with cows kept in byres. The authors believe that deficiencies in milk yield and general health frequently encountered in winter are not due to the low temperature but to bad feeding and husbandry at that season of the year. They therefore emphasize how essential it is that the animals should be well nourished and in good condition during the winter. The influence of climatic factors on the body temperature, respiration rate, flow of saliva and other physiological functions in cattle of different herds has been thoroughly investigated by Bonsma and his colleagues(328, 329). The data, which are well illustrated with maps and graphs cannot be adequately discussed in the small space available here. The failure of high-grade European cattle to withstand hot climates satisfactorily has been discussed by French(330).

Butterfat percentages in Arizona(331) have been found to vary with the season from 4·02 in April to July to 4·40 in December. Records kept(332) in 1939–40 for over 11,000 cows in Western Australia have shown that cows calving during March, April and May produced approximately 230 lb. of butterfat whereas for those calving after May the butterfat production was decreased, reaching a minimum of 190 lb. for those calving in October. An average of 200 lb. was attained again in lactations which began in December. For Red Poll and Friesian cows in Victoria(333) it has been found that cows calving between April and September produce more milk and butterfat than cows calving between October and March.

The length of the dry period is an important factor in attaining maximum milk production. In the paper just cited the length of the gestation period, the birth weights of calves and the milk and butterfat production of Red Poll cattle are compared with those of Friesian cattle. Friesians appear to require a dry period of at least 3 weeks for maximum efficiency and production whereas Red Polls appear to require 6 weeks. For Ayrshire cows in South Australia Johnston(334) recommends a dry period of 3 months and stresses that much harm may result from milking a heifer for too long a period in her first lactation. From 126 records made during 1932 to 1938 in Illinois, Yapp(335) has concluded that increasing the length of the dry period from between 20 and 39 days to between 80 and 99 days resulted in the average milk yield increasing in the following lactation from 15,120 to 17,398 lb. and the average butterfat yield from 541 to 637 lb. Information on the effect of the length of the calving interval is also available

from records kept in Michigan(336) and India(337). A detailed statistical study of records of over a thousand cows with at least three lactations has been made by Klein & Woodward(338). The changes in yield which appeared to result from variation in the length of the dry period were expressed as percentages of an average lactation yield of 10,000 lb. The optimum dry period for cows yielding 10,000 lb. of 4% fat-corrected milk and calving once a year was found to be 55 days. It is recommended that if this optimum cannot be adhered to, a longer rather than a shorter dry period is advisable. Kaleff(339), on the other hand, concludes from a statistical analysis of records for 290 cows and sixty-three buffalo cows that a *short* interval between calving and service dates has a favourable influence on lactation and on the general health and commercial value of the animal. Kaleff outlines the importance of early service, assuming that it has a very favourable effect on the endocrine glands which are particularly concerned with reproduction and lactation in the cow.

The effect of age on milk-producing capacity has been investigated by a number of workers. Dickerson & Chapman(340) divided the records for two large Holstein herds into two groups in which the age at the first calving ranged from less than 24 months to more than 36 months. Analysis of the records indicated that breeding heifers as early as 26 months was not detrimental to reproduction or growth rate and resulted in a slight but definite increase in lifetime production efficiency. Late calves were older at disposal, but had no longer productive lives than early calves. Early calving was found both by Dickerson and Chapman in America and by Hansson in Sweden(341) to have no effect on the size ultimately attained by the cow. Hansson found that *total* fat production decreases greatly with increase in age at first calving and that the best and most economical age for first calving in the Swedish Red and White breed is 26–28 months.

The influence of the age of the cow on milk and milk-fat production has been studied for various German breeds by Lange(342), who compares his results with those for British and American breeds. Fat percentage was highest in the first or second lactation and milk yield in the fifth to seventh. The occurrence of the maximum milk yield at the age of 6–7 years in Red Polish cattle has been reported by Ocetkiewicz(343), who found the fat percentage to be very little affected by age.

The effect of frequency of milking on milk yield has frequently been discussed. In some recent experiments Ludwick, Spielman & Petersen(344) milked the two halves of the udders of five Guernsey cows separately over a period of 5 months. One half of each udder was milked three times and the other half twice each day, the treatment being reversed every fortnight. The more frequent milking resulted in an increase in yield of 16%. In another recent investigation(345) one cow and two heifers were tested over 12 days. One milking per day was found to cause a 26·4% decrease in milk yield as compared with two milkings per day, whereas three milkings increased the yield by 17%. Ludwin(346) has found that when the level of production is considered, persistency of lactation is increased with more frequent milking, but if the level of production is not considered, persistency is not affected by the frequency of milking. Some objections, which the author discusses, could well be made to the methods by which he reaches these conclusions.

Work was cited on p. 209 which showed that suckling was important in the ejection of milk, but it is also a factor which affects the secretion process itself possibly by stimulating the formation of lactogens by the pituitary. This was shown in experiments by Meites & Turner(347) in which the lactogen content of the pituitaries was estimated in one group of lactating rabbits which were allowed to nurse their young and in another group in which nursing of the young was not permitted. In both groups, after parturition, there was an abrupt rise in lactogen content to a maximum on the fifth day, but for the suckled rabbits the lactogen content was 66% greater than for those which were not suckled. In the non-suckled group there was subsequently a very much more rapid decline in lactogen content. Meites & Turner(124), in experiments on rats, also explain the dependence of lactation on regular suckling as being due to the stimulation of the pituitary to secrete lactogens, for a marked decrease in pituitary lactogen occurs when suckling ceases. The effects of suckling on the mammary glands of rats has been discussed by Weichert(348), who found that the glands associated with unsuckled teats showed rapid regression compared with the suckled glands even when prolactin was injected. The great

importance of suckling in the development of milk secretion in the first lactation in humans has been stressed recently by Stewart & Pratt(349).

(5) *Other miscellaneous publications*

Papers have recently been published on the biology of the buffalo and factors affecting its milk production, milk composition and general physiology(350, 351). The production of milk by sheep in Sicily and Sardinia has also been discussed(352, 353). The composition of the milk of monkeys (*M. mulatta*) has been shown(354) on the average to resemble human milk in its low content of protein (2·1%) and ash (0·26%) and in its high content of lactose (5·9%).

The method of keeping records and the value of short-time records for predicting milk yields have been discussed by Cannon *et al.*(355, 356), by Reece(357) and by Kennedy & Seath(358).

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